NOVEL FUNCTIONS FOR THE PREGNANE X RECEPTOR INCLUDE REGULATION OF mRNA TURNOVER AND INVOLVEMENT IN COLON CANCER PROGRESSION

A Thesis

by

NAVADA LORRAINE EAGLETON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Toxicology

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Approved by:	
Chair of Committee,	Yanan Tian
Committee Members,	Stephen Safe
	Weston Porter
	Alice Villalobos
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ABSTRACT

Novel Functions for the Pregnane X Receptor include Regulation of mRNA Turnover and Involvement in Colon Cancer Progression. (August 2010)

Navada Lorraine Eagleton, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Yanan Tian

To understand the mechanisms of transcriptional regulation of PXR, we performed yeast two-hybrid screenings to search for PXR-interacting proteins in a human liver cDNA library using the PXR ligand binding domain as the bait. More than one million independent clones were screened. One positive clone was a partial cDNA of CNOT2 (amino acid 183-540). CNOT2 is a component of CCR4-NOT that is a multi-subunit protein complex highly conserved from yeast to humans.

Using a mammalian two-hybrid system in CV-1 cells and GST-pull down assays, we confirmed the direct interaction between PXR and CNOT2 and mapped the specific domains of association. In HepG2 cells, over expression of CNOT2 suppressed the PXR-regulated luciferase reporter gene activity. siRNA knockdown of CNOT2 potentiated PXR-transcriptional activity. These results strongly suggest that the CCR4-NOT complex is significantly involved in transcriptional regulation of PXR.

The immuno-precipitated CNOT2 complex contained deadenylase activity as determined by an *in vitro* RNA decay assay. The presence of transfected PXR inhibited the cNOT2-associated deadenylase activity, as demonstrated by poly(A) tail PCR. Cellular localization of PXR and cNOT2 by immuno-fluorescence microscopy indicates

that the interaction might occur within Cajal Bodies. Taken together, these results suggest that PXR regulates the mRNA turnover through direct interaction with the NOT2 component of the CCR4-NOT complex.

PXR is also involved in colon cancer progression. Our results indicate that the evolutionarily conserved PXR protects organisms from carcinogenesis by inhibiting tumor growth as well as eliminating carcinogenic substances. Our laboratory proposes that pregnane X receptor has an important role in maintaining the balance of cells progressing through the cell cycle. *In vitro* and *in vivo* experiments demonstrate expression of PXR in colon cancer cells slows the progression of tumor formation. Colony growth of the PXR-transfected HT29 cells was suppressed in soft agar assay.

In the xenograft assay, the tumor size formed in nude mice was significantly suppressed in HT29 cells stably transfected with PXR (310 mg±6.2 vs. 120 mg±6, $p<0.01$). The number of Ki-67 positive cells were significantly decreased in PXR-transfected HT29 xenograft tumor tissue compared vector-transfected HT29 controls ($p<0.01$) as determined by immuno-histochemistry suggesting that PXR inhibits proliferation of colon cancer cells. Results of flow cytometry analysis indicated that PXR-transfection in HT29 cells caused G_0/G_1 arrest. The growth inhibitory effects of PXR are likely mediated through the E2F/Rb-regulated check point since E2F1 nuclear expression was significantly inhibited by PXR over expression.

DEDICATION

A fortune cookie once told me “Every wise man starts out by asking questions.” I believe that being curious is a requirement of a truly brilliant scientist. This thesis is dedicated to family and friends that have always been there to answer my questions no matter what I was asking. This is only one step on the pyramid of knowledge I am eagerly climbing.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Yanan Tian, for introducing me to research as an undergraduate and allowing me to cultivate my love for research in his lab. I also thank the other members of the committee: Dr. Stephen Safe, Dr. Weston Porter and Dr. Alice Villalobos, for their guidance and support throughout the course of this research.

I am extremely grateful to Sui Ke for her help with experiments; without her nothing in the lab is possible. I greatly appreciate the members of the Tian lab for their help in my research, their friendship and enlightening conversations about issues domestic and foreign - Ying Xie, Dr. Nengtai Ouyang, Dr. Xinsheng Gu, Dr. Duan Liu, Hongmei Cui, Dr. Henrui Yao, Dr. Yanqun Xiang, Dr. Jessica Epple-Farmer.

In general, I would like to recognize the Texas A&M Toxicology Program in the College of Veterinary Medicine. This is a solid program and Kim Daniel has helped me as an undergraduate traveling with the graduate students to my first SOT meeting. She has continued to be supportive through my graduate studies. I have also made wonderful friends and colleagues, for whom I have the utmost respect. These include the members of Drs. Phillips', Porter's, Safe's, Sayes', and Donnelly's laboratories. Thank you for making the time spent working on my thesis fun and enjoyable.

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INTRODUCTION AND BACKGROUND

Central to our understanding of xenobiotic/drug metabolism and avoidance of adverse drug responses including drug-drug interaction is to delineate the underlying mechanisms of how drugs are metabolized and the cofactors that modulate this activity. The importance of understanding the effects of combinations of chemicals has increased due to current therapeutic treatments and the ever changing chemical environment produced from the growing human population. Complications that arise from drug-drug interactions can result in decreased clinical efficacy of the drugs or increase toxicity. Adverse drug reactions are the 4th largest cause of mortality in the western world (Plant, 2007). It is crucial to understand chemical interactions and drug-drug interactions to accurately predict human biological responses. Regulation of diseases requires the balance of activating and inhibiting various transcription factors. Therefore, it is very important that the study of the relationships among genetic polymorphisms, cancer susceptibility, environmental exposures and toxicity strive to achieve a comprehensive understanding of these complex mechanisms and the associated implications for prevention and treatment of human disease. The molecular pathways that govern disease are coordinately regulated by some of the same processes involved in metabolism and clearance of internal and external chemicals in the human body.

This thesis follows the style of *British Journal of Cancer*.

Drug metabolism and detoxification

Expression of drug metabolizing enzymes is highly adaptable in response to inducing compounds. Protein levels increase or decrease in response to exposure of chemical agents. Understanding these molecular mechanisms helps to predict xenobiotic interactions that could potentially have toxic effects. Nuclear receptors are responsible for communications from an internal or external chemical stimulus to target genes required to metabolize or expel the compound from the body. The role of nuclear receptors has evolved from their prototypic function in bacteria to a more complex version in humans and higher mammals.

In general, drug metabolism and detoxification begins with a nuclear receptor responding to specific ligands that result in translocation to the nucleus and induction of the target gene by binding to drug response elements. Once the target gene is expressed its corresponding enzyme can bind the substrate and begin metabolism. Phase I and Phase II enzymes usually act together to metabolize the compound and ready it for excretion. However, Phase II of metabolism/detoxification is not always preceded by Phase I (Klaassen and Watkins, 2001). Both groups of enzymes can be found in microsomes in the endoplasmic reticulum, the organelle responsible for protein and lipid maintenance. However many Phase I and Phase II enzymes can be found in the cytosol or mitochondria. (See Table 1 and 2 for a complete list of enzymes and their cellular locations.) Cytochrome P450 enzymes, or CYPs, are Phase I drug metabolizing enzymes. Cytochrome P450 enzymes act through hydrolysis, reduction, and/or

oxidation making the compound more polar and ready for action by Phase II DMEs (Klaassen and Watkins, 2003).

TABLE 1: Phase I biotransformation of xenobiotics. These enzymes are responsible for Phase I biotransformation of xenobiotics and can be found in various cellular locations.

Reactions	Enzymes	Locations
Hydrolysis	Esterase	Microsomes, cytosol, lysosomes, blood
	Peptidase	Blood, lysosomes
	Epoxide hydrolase	Microsomes, cytosol
Reduction	Azo- and nitro- reductase	Microflora, microsomes, cytosol
	Carbonyl reductase	Cytosol, blood, microsomes
	Disulfide reductase	Cytosol
	Sulfoxide reductase	Cytosol
	Quinone reductase	Cytosol, microsomes
	Reductive dehalogenase	Microsomes
Oxidation	Alcohol dehydrogenase	Cytosol
	Aldehyde dehydrogenase	Mitochondria, Cytosol
	Aldehyde oxidase	Cytosol
	Xanthine oxidase	Cytosol
	Monoamine oxidase	Mitochondria
	Diamine oxidase	Cytosol
	Prostaglandin H synthase	Microsomes
	Flavin-monooxygenase	Microsomes
	Cytochrome P450s	Microsomes

Phase II drug metabolizing enzymes detoxify chemicals through conjugation, glucuronidation, sulfation, acetylation, and/or methylation and this makes the compound more water soluble and ready for excretion (Klaassen and Watkins, 2003).

TABLE 2: Phase II biotransformation of xenobiotics. These enzymes are involved in Phase II biotransformation of xenobiotics and are found in various cellular locations.

Reaction	Enzymes	Locations
Glucuronide conjugation	UDP-glucuronosyltransferases	Microsomes
Sulfate conjugation	Sulfotransferases	Cytosol
Glutathione conjugation	Glutathione S-transferases	Cytosol, microsomes
Amino acid conjugation	Acyl-CoA:amino acid N-acyltransferase	Mitochondria, microsomes
Acylation	Arylamine N-acetyltransferases	Mitochondria, Cytosol
Methylation	Methyltransferases	Cytosol, microsomes, blood

Metabolism and detoxification of endogenous and exogenous compounds is a necessary biological process; unfortunately many of the exogenous compounds that require metabolism also become metabolically activated to give carcinogens.

Pregnane X receptor and nuclear receptor super family

The pregnane X receptor (PXR, NR1I2, also known as steroid X receptor (SXR) in humans) is an evolutionarily conserved receptor that belongs to the nuclear hormone

receptor (NR) superfamily. PXR was first cloned in mice by the Kliewer lab in 1998 (Kliewer *et al,* 1998). Since then PXR homologs have been verified in rats, dogs, cows, primates, humans and zebrafish (NCBI Homologene). The nuclear receptor superfamily is the largest family of ligand-activated transcription factors; in total there are 48 NRs in humans (Zhang *et al,* 2004). [Table 3] It must be noted that members belonging to the nuclear receptor superfamily are not the only transcription factors responsible for regulating CYP enzymes. Another important group of transcription factors are those belonging to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) DNA binding motif family. The aryl hydrocarbon receptor (AhR) and its partner aryl hydrocarbon receptor nuclear translocator (ARNT) are bHLH/PAS transcription factors. The AhR-ARNT heterodimer is responsible for transcriptional regulation of cytochrome P450 1A1 (CYP1A1), a protein that metabolizes many environmental toxicants, such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines and heterocyclic amines.

TABLE 3: Human nuclear receptors. These proteins respond to respective ligands and initiate expression of proteins responsible for many biological processes including drug metabolism and detoxification (Robinson-Rechavi *et al,* 2001).

Nuclear receptor	***Natural ligand***	***Classical Nomenclature***
Thyroid hormone receptor α/β	Thyroid hormone	NR1A1, 1A2
Retinoic acid receptor α/β/γ	Vitamin A, related compounds	NR1B1, 1B2, 1B3
Peroxisome proliferator-activated receptor α, δ, γ	Fatty acids, prostaglandins	NR1C1, 1C2, 1C3
RAR-related orphan receptor α/β/γ	Cholesterol	NR1F1, 1F2, 1F3
Liver X receptor α/β	Oxysterol	NR1H3, 1H2
Farnesoid X receptor	Oxysterol	NR1H4
Vitamin D receptor	Vitamin D	NR1I1
Pregnane X receptor	Pregnanes ,xenobiotics	NR1I2
Constitutive androgen receptor	Androstane	NR1I3
Hepatocyte nuclear receptor-4 α/γ	Fatty acids	NR2A1, 2A2
Retinoid X receptor α/β/γ	Retinoids	NR2B1, 2B2, 2B3
Testicular receptor 2/4	N/A	NR2C1, 2C2
Estrogen receptors α/β	estrogen	NR3A1, 3A2
Estrogen-related receptor α/β/γ	Sex hormones	NR3B1, 3B2, 3B3
Glucocortoid receptor	Cortisol	NR3C1
Mineralocorticoid	Aldosterone	NR3C2
Progesterone receptor	Progesterone	NR3C3
Androgen receptor	Testosterone	NR3C4
Steroidogenic factor 1	Phospholipids	NR5A1
Nerve growth factor IB-like	N/A	NR4A1, 4A2, 4A3
Germ cell nuclear factor	N/A	NR6A1
DAX (dosage-sensitive reversal on X chromosome)	N/A	NR0B1

Each nuclear receptor plays an important role in mammalian physiological functions by regulating transcriptional activity involved in processes including reproduction, differentiation, development, metabolism, metamorphism, and homeostasis

(Gronemeyer *et al,* 2004). For example, PXR regulates these processes by inducing expression of multiple target genes upon ligand activation; including cyp2b, cyp3a, multidrug resistance transporter (mdr1), and cyp2c9 (Sahi *et al,* 2009). MDR1 is a Phase III drug transporter that protects cells from toxicity by quickly pumping drugs from the cells (Synold *et al,* 2001). All PXR-regulated genes products provide hepato-protection through drug metabolism and clearance.

Most nuclear receptors have the same basic structure. [Figure 1] The DNA binding domain (DBD) and ligand binding domain (LBD) are the major domains responsible for the transcriptional regulation activity of nuclear receptors. Located within the DBD are two cysteine coordinated zinc-fingers that are responsible for DNA binding and dimerization. The LBD is involved in receptor dimerization and interactions with co-regulators (Mohan and Heyman, 2003). Other regions important to the functionality of NRs include the transcriptional activation function 1 and 2 (A/B and F). These domains are located at the N-terminal and C-terminal regions respectively. The hinge domain is located between the DNA binding domain and ligand binding domain and functions to allow conformational changes. Upon ligand binding, nuclear receptors undergo conformational changes resulting in the release of co-repressors, recruitment of co-activators and the induction of transcription (Xu *et al,* 1999).

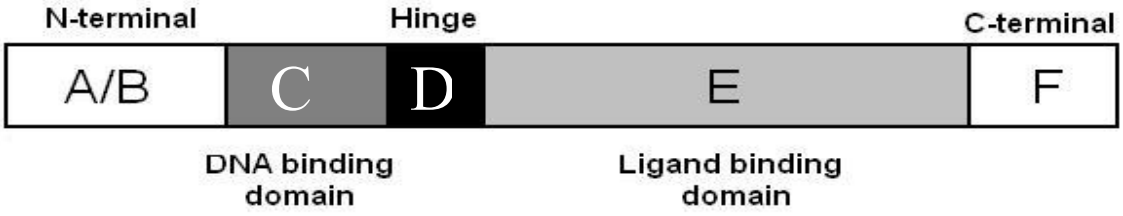

FIGURE 1: Nuclear receptor domains. The most important domains for the activity of a nuclear receptor are the DNA binding domain and the ligand binding domain. The DBD allows the nuclear receptor to act as a transcription factor and the LBD interacts with a wide range of endogenous and exogenous ligands. (Garcia *et al,* 2003)

Compared to other nuclear receptors, PXR is extremely divergent across species (Ekins *et al,* 2008). Structure of the ligand binding domain varies greatly resulting in activation of PXR by different compounds in different species. For example, rifampicin is an extremely effective activator of human PXR but has little effect on mouse PXR; whereas the inverse is true for pregnenolone-16α-carbonitrile (PCN) (Ekins *et al,* 2007).

Pregnane X receptor ligand activation

PXR is a ligand-dependent transcription factor that is activated by a wide range of endogenous and exogenous compounds and substances. PXR was once considered to be an orphan nuclear receptor because at the time no natural ligand had been discovered; however 5β-pregnane-3,20-dione was found to naturally activate PXR (Mohan and Heyman, 2003). PXR is now considered an adopted nuclear receptor and PXR ligands are hydrophobic, small molecules. Human PXR ligands range from endogenous compounds such as bile acids (eg. lithocholic acid), many androstane/estrane steroids to exogenous compounds such as phenobarbital, clotrimazole, rifampicin, and vitamins like

vitamin D, β-carotene and herbal supplements such as hyperforin the active component in St. John's wort (Ekins *et al,* 2007; Goodwin *et al,* 1999; Ekins and Erickson, 2002).

After ligand activation, PXR heterodimerizes with retinoid acid receptor (RXR) and the complex translocates to the nucleus where it binds the xenobiotic response element (XRE) or xenobiotic response enhancer module (XREM) on the promoter of its target genes (Kliewer, 2003). PXR-RXR binds to a recognition sequence on the target gene that consists of two half sites of AGGTCA arranged as either an everted repeat separated by six nucleotides (ER6) or a direct repeat separated by three (DR3) or four (DR4) nucleotides (Handschin and Meyer, 2003). Located within the distal enhancer region of CYP3A4 are two ER6-type PXR response elements located -7738bp to -7717bp and -7698 to -7682. [Figure 2] Nuclear receptor binding sites typically have high levels of promiscuity because the binding site is not specific for one receptor. For example, competitive binding is seen between PXR and CAR. Both can bind the same xenobiotic response elements on CYP2B and CYP3A genes; however, there is a clear ligand preference for activation of either nuclear receptor to the corresponding preferential target gene (Xie *et al,* 2000).

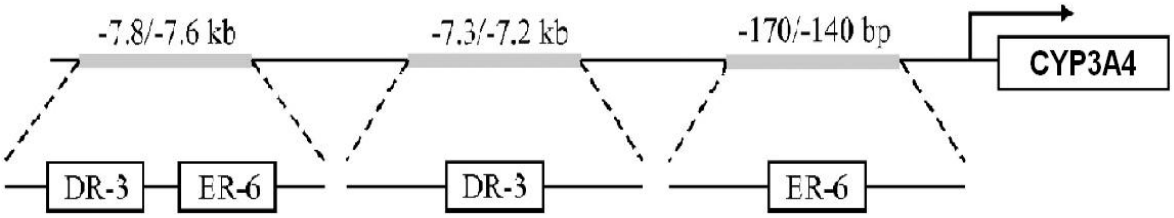

FIGURE 2: Xenobiotic responsive elements of cyp3a4 gene. Consensus sequences within the promoter and enhancer regions of cyp3a4 are responsible for nuclear receptor binding and subsequent gene expression. (Handschin and Meyer, 2003)

Importance of cytochrome P450 enzymes

The wide distribution of cytochrome P450s indicates that they are critical for survival. These enzymes existed in organisms prior to the divergence of eubacteria from eukaryotes, suggesting that these enzymes were responsible for important life functions long before animal-plant divergence (Nebert and Dieter, 2000). Cytochrome P450 enzymes (CYPs) are Phase I drug metabolizing monooxygenases that are found in all organisms, ranging from bacteria to humans. Phase I enzymes are responsible for the biotransformation of endogenous and exogenous chemicals, typically by adding hydroxyl groups to the compound to make them more polar. There are 57 CYPs found in humans. All drug metabolizing enzymes have endogenous compounds as natural ligands, but are also responsible for metabolism of over-the-counter medications and pharmaceuticals. Drug metabolizing enzymes play a role in the synthesis and degradation of every known non-peptide involved in ligand-dependent transcriptional processes that affect growth, differentiation, apoptosis, homeostasis and neuroendocrine functions (Nebert and Dieter, 2000).

For the metabolism of xenobiotics in humans, the most notable gene target is the cytochrome P450 3A family, which includes 3a4, 3a5, 3a7 and the recently identified 3a43 (LeCluyse, 2001; Luo *et al,* 2004). Isoform protein expression levels vary greatly between individuals due to environmental and genetic differences that have been accentuated over thousands of years of human evolution (Thummel and Wilkinson, 1998). Most cytochrome P450 genes are present in clusters on chromosomes, probably originating from gene duplications or other chromosomal alteration events. Human

cyp3a is found on chromosome 7 location 7q21-.1 arranged in order 3a4, 3a7, 3a5. [Figure 3] Each protein variant that is produced is composed of 13 exons forming approximately a 2 kb transcript (Finta and Zaphiropoulus, 2000).

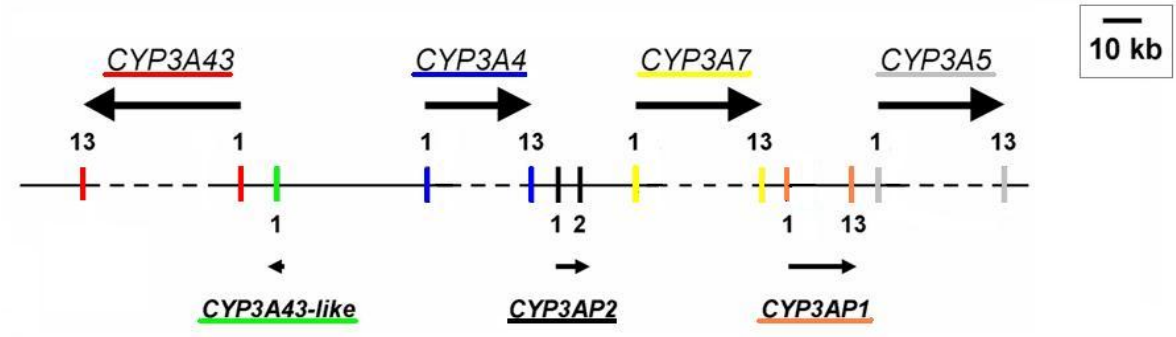

FIGURE 3: Cytochrome P450 3a gene cluster and transcripts. The cyp3a gene produces four protein isoforms each consisting of 13 exons. Cyp3a also contains three pseudo genes. (Finta and Zaphiropoulus, 2000)

CYP3A5 expression is limited to only one fourth of human livers; protein levels are about the same or slightly higher than 3A4 in populations that express both isoforms (Krusekopf *et al,* 2003). CYP3A7 is the predominant form found in fetal liver; however, expression decreases rapidly after birth in an inverse relationship to 3A4 (Plant, 2007). CYP3A7 also is highly expression in endometrial and placental tissue, possibly indicating a mechanism of fetal protection (Sim *et al,* 2005). Cyp3a43 was recently cloned in 2001 and is expressed at low levels with low metabolic activity (Daly, 2006). Krusekopf *et al.* (2003) demonstrated that most substrates of CYP3A4 can be metabolized by the other 3A isoforms, but for most substrates CYP3A4 has higher metabolic activity.

CYP3A4 is the most abundant drug metabolizing enzyme in the liver. Over 50% of clinical drugs are subject to metabolism by this enzyme (Kolars *et al,* 1994). Expression of proteins involved in drug metabolism and detoxification have been found in skin, lung, nasal mucosa, eye, gastro-intestinal tract, kidney, adrenal gland, lymphocytes, pancreas, spleen, heart, brain, testis, ovary, placenta, plasma, erythrocytes, platelets and aorta (Gram, 1980; Farrell, 1987; Klotz, 1994).

Protein-protein interactions

The recent focus of our laboratory has been identifying novel roles for the pregnane X receptor or PXR, an important transcriptional regulator of drug metabolism. For example, our group has demonstrated- (1) PXR interacts with PRMT1 to regulate histone methylation (Xie *et al,* 2009), (2) PXR is involved in mediating DNA repair inflicted from ultraviolet light and benzo(a)pyrene (Naspinski *et al,* 2008), (3) PXR regulated pathways that interact with the inflammatory mediator NF-κB resulting in suppression of drug metabolizing enzyme activity (Xie and Tian, 2006; Gu *et al,* 2006). To further analyze the PXR interacting protein factors, we performed yeast two hybrid assays using PXR-ligand binding domain as the bait. After screening over a million clones from the human cDNA liver library we identified a partial clone of component NOT2, part of the CCR4-NOT complex, as an interactive partner of pregnane X receptor. The research in this thesis continues to expand the role of PXR regulation beyond drug metabolism to encompass functions of RNA regulation and mRNA turnover through interactions with the human CCR4-NOT complex.

Our laboratory has also identified a role for PXR in colon cancer progression. PXR is actively involved in tumor promotion and progression by affecting pathways involved in not only carcinogen detoxification but also associates with cell cycle check point proteins and factors involved in tumor invasion and metastasis. Novel physiological functions of PXR have been reported indicating that PXR plays roles in physiological and patho-physiological processes extending beyond metabolism and detoxification of xeno/endobiotics. These novel functions are likely based on cross-talk mediated through protein-protein interactions. The results presented in this thesis and previous publications from our laboratory demonstrate that the PXR is a dynamic multi-functional nuclear receptor that is involved in many biological processes beyond its primary role in drug metabolism.

CCR4-NOT is a multi-protein complex

CCR4-NOT was one of the most intriguing PXR interactive partners identified in the human liver cDNA library screens. An evolutionary conserved complex, CCR4-NOT is present in most eukaryotic cells. In *Saccharomyces cerevisiae*, CCR4-NOT is the primary complex responsible for mRNA modification and degradation (Cuthbertson and Blackshear, 2008). There are two major forms of the complex- a 1.0 MDa complex and a 1.9 MDa complex (Denis and Chen, 2003). The masses of the complex vary due to protein factors associating with CCR4-NOT core components for many of its biological functions. The complex is composed of nine core protein subunits- CCR4, Caf1, Caf40, Caf130 and NOT1-5. [Figure 4]

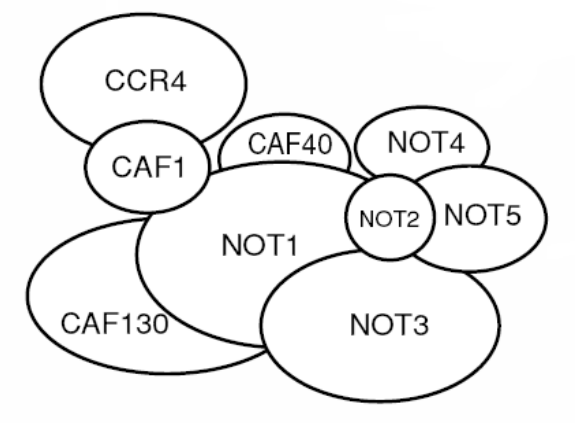

FIGURE 4: CCR4-NOT complex. The multi-protein complex is composed of nine subunits. (Denis and Chen, 2003)

Clear modulation of the complex provides various functions by allowing the different components to come into contact with their respective interacting partners. There is still debate as to whether the components of CCR4-NOT can act individually or only as a complex. In yeast it was shown that CCR4-Caf1 and NOT2-5 components compose two very distinct modules that are both physically and functionally separated, with NOT1 as the bridge that holds the complex together (Bai *et al,* 1999). NOT1 is the scaffolding protein and the only protein component that is necessary for formation of the complex (Cuthbertson and Blackshear, 2008). NOT2 and NOT3 are similar in structure and affect transcriptional activity through interactions with key transcription factors. NOT4 is an E3 ubiquitin ligase involved in protein degradation (Albert *et al,* 2000); and NOT5 interacts with TFIID affecting positioning on the promoter (Lenssen *et al,* 2007). The NOT components are primarily responsible for protein-protein interactions that positively or negatively affect regulation of gene expression, whereas CCR4, CAF1, CAF40 and CAF130 are responsible for RNA deadenylation.

Examples of the many roles of CCR4-NOT components include involvement with the exome, histones, oxidative phosphorylation, vacuole and cell wall by up or down regulation of genes involved in these processes (Azzouz *et al,* 2009). In HeLa cells, Garapaty *et al.* (2008) demonstrated that the CCR4-NOT complex can act as an activator through interaction with NRC-interacting factor NIF-1. Garapaty's group demonstrated that CCR4-NOT mediates activation through association with the ligand binding domain of nuclear hormone receptors. NOT components function as transcriptional repressors through restricting TFIID access to the promoter (Cui *et al,* 2008, Collart *et al,* 1993, 1994, 1996). Every component of the CCR4-NOT complex has a specific function based on its individual protein-protein interactions.

RNA transcript deadenylation appears to be one of the most biologically significant roles for the CCR4-NOT complex. Components of the CCR4-NOT complex are involved in cell proliferation and apoptosis through interaction with the CDK inhibitor p27 and antiproliferative protein Tob, respectively, by modulating the rate of deadenylation (Morita *et al,* 2007; Miyasaka *et al,* 2008). When HEK293T cells are depleted of CCR4 protein, the deadenylase activity of the complex is lost resulting in cell growth impairment due to an increase in p27 mRNA (Morita *et al,* 2007). Deadenylase activity of the complex is also decreased in NIH3T3 cells through interaction of CNOT1 with the antiproliferative protein Tob (Miyasaka *et al,* 2008). Aslam *et al,* found that the CCR4-NOT deadenylase subunits CNOT7/Caf1a and CNOT8/Caf1b have overlapping roles and modulate cell proliferation (Aslam *et al,*

2009). The NOT components do not exhibit deadenylase activity but can direct RNA regulation through their various interactive partners.

Specific functions of CNOT2

The second NOT component, CNOT2, is a 26 kDa protein and the smallest component of the CCR4-NOT complex. However, it plays an important role in maintaining proximity of components NOT3, NOT4 and NOT5 with the rest of the complex (Denis and Chen, 2003). Therefore, CNOT2 is also essential for the complex to function in its entirety. Experiments in yeast show that mutations within CNOT2 dramatically affect cellular processes such as maintaining integrity of the exome, vacuole, and oxidative phosphorylation (Azzouz *et al,* 2009).

CNOT2 is typically thought of as a repressor of transcriptional activity (Traven *et al,* 2005). NOT or negative on TATA indicates the NOT components can negatively regulate transcriptional activity at the promoter level. The NOT Box domain is primarily responsible for repression by CNOT2. However, repression by CNOT2 is greater than the NOT Box domain acting alone (Zwartjes *et al,* 2006). For example, CNOT2 repression is enhanced when co-expressed with SMRT or NCoR in combination with HDACs (Jayne *et al,* 2006). CNOT2 makes contact with ADA2, a component of the SAGA complex which is involved in cellular stress responses, resulting in repression of SAGA-responsive genes (Russel *et al,* 2002). Shi and Nelson (2005) screened the human fetal brain cDNA library to find that CNOT2 and CDK11 interact with each other.

RNA processing

The central dogma of molecular biology, first described by Francis Crick in 1958, indicated that only one middle man was necessary to pass genetic information from step to step in gene expression. However, he also pointed out that the flow was only one-way. Proteins could not be transformed into nucleic acids in a reverse reaction. [Figure 5] Ribonucleic acids (RNA) play the intermediate messenger between DNA and protein.

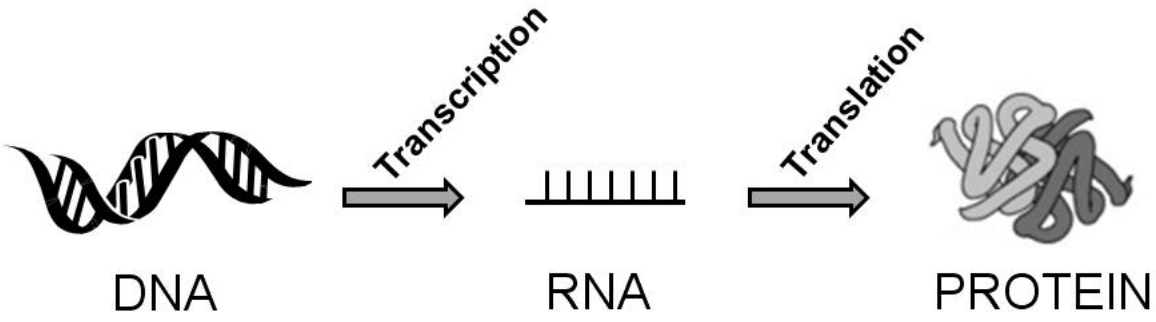

FIGURE 5: Diagram of the central dogma of molecular biology

When the human genome was decoded in 2000, scientists discovered that over 95% of our genome was "junk DNA"; in other words it did not specifically encode for a protein. The majority of noncoding sequences are relics left from millennia old translocations, duplications and other chromosome alterations. However, the term junk DNA is becoming obsolete due to new evidence showing that the junk regions play a major role in gene regulation, specifically at the RNA level (Bernstein *et al,* 2001; reviewed in Shi, 2003). For example, in some cases RNA interference (RNAi) influences the outcome of translation by inactivating mRNA transcripts before they are able to

come into contact with a ribosome. These bits of RNA come from RNA duplexes that are spliced or diced.

RNA modifications occur within the nucleus and the cytoplasm. After transcription the newly produced RNA transcript (pre-mRNA) is transported to Cajal bodies where modifications occur that will stabilize the transcript for transport into the cytoplasm where translation occurs (Rippe, 2007). The 5' end is capped and 3' poly(A) tail is added to stabilize the mRNA transcript. The poly(A) tail aides in stabilization by elongating the end of the transcript with repeated adenine nucleotides. This process keeps the 3' end guarded from exonucleases that could possibly degrade the transcript before it reaches a ribosome for translation (Meyer *et al,* 2004). Deadenylation of the 3' poly(A) tail of the of the mRNA transcript decreases stability, which makes the transcript easier to degrade by exonucleases. The CCR4-NOT complex has been shown to be actively involved in mRNA deadenylation; multiple assays exist to determine degeneration of the poly(A) tail (Meijer *et al,* 2007; Salles *et al,* 1999).

Cajal bodies (CBs) were named after the Spanish scientist Ramon Cajal who discovered nucleic foci while studying the neuron in the 1920s. Coilin is a structural protein found exclusively in Cajal bodies. An unconventional CCR4-Caf1 deadenylase complex was found to concentrate in CBs and shuttle to and from the nucleus (Wagner *et al,* 2007). Outside of the nucleus there are multiple structures involved in RNA modifications- gems, p-bodies, GW182 or Dcp-containing bodies. P-bodies are important for RNA decay following protein synthesis; mRNA turnover is extremely important for regulating gene expression. Other cytoplasmic bodies function to remove

aberrant mRNA before translation to eliminate the risk of producing deleterious proteins. Cougot *et al.* (2004) showed that CCR4 protein colocalizes with Dcp16, which is active in mRNA decay. All data indicate that nuclear and cytoplasmic foci are extremely dynamic structures. They require ongoing transcription to form; when transcription or translation is inhibited these foci will dissolve.

CCR4-NOT complex has a role in most biological processes due to the versatility of protein-protein interactions of the core components of the complex. CNOT2 and PXR directly interact to suppress transcriptional activity of PXR and to direct the protection of deadenylation of cytochrome P450 3A4 mRNA.

Pregnane X receptor is involved in colon cancer

Many endogenous and exogenous compounds become carcinogenic after metabolism and detoxification has occurred (Ekins *et al,* 2007). PXR also has an innate role in normal liver cell regeneration and repair. Dai *et al.* (2008) showed that hepatocyte proliferation in PXR null mice was inhibited after partial hepatoectomy. Their results indicate that PXR is required for normal progression of liver regeneration by modulating lipid homeostasis and regulating hepatocyte proliferation. Increased expression of PXR may increase the detoxification capability of cancer cells resulting in an increased resistance to anticancer agents (Matic *et al,* 2007; Nittke *et al,* 2008; Chen *et al,* 2007) and PXR plays a role in sensitization of cells for apoptotic responses (Zhou *et al,* 2008). Even though these studies have conflicting results, it is clear that PXR has a role in cancer. The same processes that govern cell regeneration regulate tumor formation.

Multistage carcinogenesis

Multistage carcinogenesis involves three main steps: initiation, promotion, and progression. The initiation step begins with a carcinogen coming into contact with the target system triggering a mutation in a target cell. This can occur through bioactivation of a potential carcinogen during metabolism by Phase I or detoxification by Phase II enzymes. It requires more than one type of insult to progress to cancer because the initiator is usually administered in low doses over a period of time. Knudson's multiple hit hypothesis accounts for the different types of damage a cell can withstand before progressing from initiation to promotion to finally, cancer progression and tumor formation (Weinberg, 2006).

For example, carcinogens are produced while cooking food. The charring or burning of food produces benzo(a)pyrene due to the combustion reaction that occurs at high temperatures. Our bodies metabolize nutrients in food and also detoxify some of the ingested chemicals. Bioactivation of toxins can result in DNA adduct formation and also RNA and/or protein adducts. However, DNA adducts are most common. A bulky adduct causes mutations that may be incorporated into DNA sequence prior to replication. Each time the cell divides it will pass on the random mutation. Usually mutations occur in areas of noncoding DNA, but sometimes they occur in tumor suppressor genes or proto-oncogenes. Key tumor suppressor genes that have altered expression in a wide range of cancers include TP53, Rb, APC, and TGFβ. Tumor suppressor genes keep normal cellular processes in check and are usually expressed at high levels. Proto-oncogenes are just the opposite; they promote cell growth and are

regulated directly or indirectly by tumor suppressor genes. Examples of oncogenes include RAS, MYC, AKT, and VEGF; these oncogenes have a major impact on tumor progression. Ultimately, multi-stage carcinogenesis is linked to the cell's ability for detoxification because the body relies on clearance of carcinogens.

Cancer progression

Many cellular pathways are involved in promoting tumor formation. Cancer is primarily a disease of aberrant signaling, and hijacking of normal biological processes that are essential for maintaining balance between proliferation and apoptosis. Cancer progression requires a combination of events to occur for a cell to be transformed (Weinberg, 2006). (1) Insensitivity to anti-growth signals occurs when the cell no longer responds to proteins that would normally halt progression of the cell cycle, if check points were not passed. (2) The cell can also gain control of its own growth signals, in effect becoming self sufficient without chemical signals from other cells. (3) Evading apoptosis allows cells with mutations in tumor suppressing genes to continue to growth and divide. (4) Mutations in genes involved in telomere maintenance can give cells limitless replicative potential. (5) Continuous blood flow is necessary to nurture a growing mass of cells. Cancer cells seek out blood streams by controlling the process of angiogenesis. (6) Cells that have achieved some of the feats mentioned above may use their new abilities for transport and invasion to other tissues to start new cancer cell colonies; this is called metastasis.

The eukaryotic cell cycle is regulated by a series of proteins that either inhibit the cycle or allow it to progress. There are check points before each step that closely

monitor DNA replication, intermittent cell growth and cell division. If the check point proteins sense any mistakes in the sequence, misalignment of required cellular machinery or incorrect pairing of chromosomes the cell cycle will arrest. Repair mechanisms are then activated before the cell cycle continues. E2F proteins and their interactive partner protein Retinoblastoma (pRb) regulate the flow of the cell cycle. E2F genes are minimally expressed during quiescence, the cell's silent state, but are induced as the cell enters the cell cycle. There are eight E2F genes that encode nine protein species; these can be classified as transcriptional activators or repressors (Trimarchi and Lees, 2002). When E2F proteins are bound to pRb, or one of its homologs p107 and p130, cell cycle progression is halted. Upon phosphorylation of retinoblastoma, EF2 is released to induce entrance into either DNA synthesis or mitosis from a phase of sentient growth. [Figure 6] In every cell cycle there is essentially a coordinated switch from the repressive to activating E2Fs that enables activation of genes involved in DNA replication and cell cycle progression (laquinta and Lees, 2007).

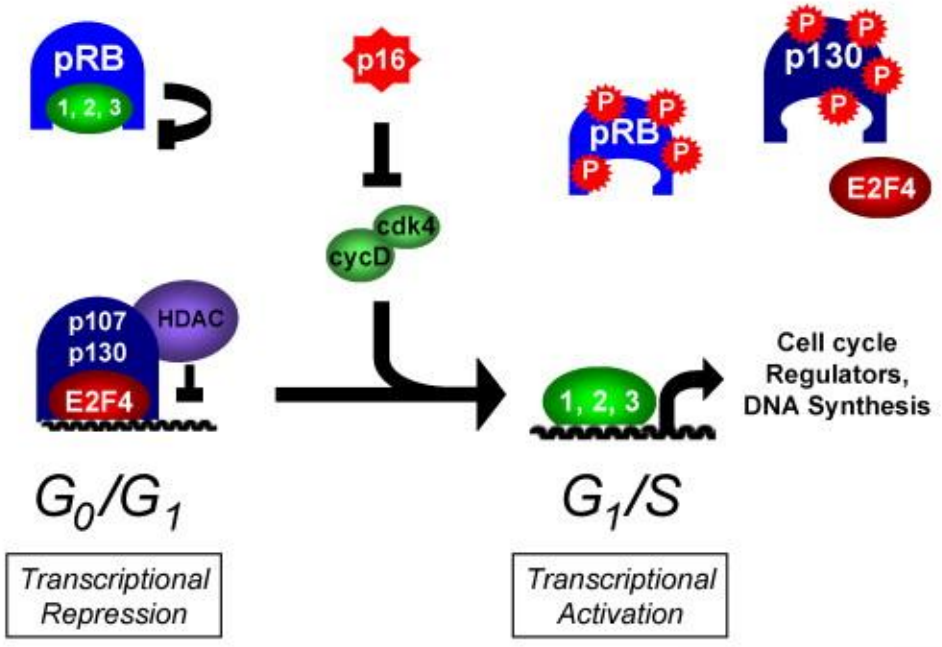

FIGURE 6: E2F and Rb proteins regulate cell cycle progression. When Rb protein is bound to E2F protein the cell cycle is halted; upon Rb phosphorylation E2F is released and acts as a transcription factor to promote cell cycle progression. (Zhu *et al*, 2005)

A series of chemokines stimulate phosphorylation of Rb/E2F, the cyclins and CDKs or cyclin-depedent kinases. The cyclin-CDK signaling is regulated by CDK inhibitors that are responding to cell cycle sensors during check points. The cell can induce apoptosis, programmed cell death, to prevent the cell from spreading future side effects produced from a cell cycle check point violation. Induction of apoptosis involves sensing cellular or environmental cues, leading to activation of intracellular signaling pathways. Activation of E2F-mediated transcription causes an increase in proteins involved in apoptosis (laquinta and Lees, 2007). The cell cycle is essentially an interconnected network of crosstalk pathways that respond to the environment to maintain homeostasis.

Pregnane X receptor's primary function is to regulate transcription of its various target genes, genes that are involved in drug metabolism and detoxification. Our laboratory has demonstrated that PXR protein regulates pathways involved in cancer progression. It is clear that receptor-regulated drug metabolism and detoxification can be causative of cancer in certain circumstances.

MATERIALS AND METHODS

Chemicals and reagents

DMSO, rifampicin, anti-FLAG M2 antibody, anti-FLAG M2-agarose affinity beads, anti-HA antibody and anti-HA agarose affinity beads were from Sigma. Isotype IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA). Nitrocellulose membranes were from Bio-Rad.

Cell culture

HepG2, CV-1, Cos7 and HT29 cells were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Sigma) and 1X antibiotic and antimycotic (Invitrogen, Carlsbad, CA). Cell lines were maintained at 37°C in 5% CO_2. PXR-HepG2 and PXR-HT29 stable transfectants were created as described in 73.

Plasmids

Plasmids expressing GST-fused PXR fragments have been created in out laboratory. DNA sequences coding different PXR fragments were PCR-amplified and subcloned into pGEX-5X-3 expression vector (Amersham Biosciences). pACT, pBIND, and pG5-luc were purchased from Promega (Madison, WI) for the mammalian two-hybrid assay. pBIND-PXR (Gal4-PXR) and pACT-CNOT2 were constructed by inserting PCR-amplified CNOT2 DNA sequence into pACT vector following the manufacturer's recommendation (Promega).

The expression vector for hPXR, pCI-hPXR was generated as follows: DNA fragment corresponding to the coding region of hPXR (amino acids 1–434) was

generated by reverse transcription-PCR using total RNA from HepG2 cells. For pCI-PXR, the PCR primers were 5'-gggaattcccaccaggaggtgagacccaaagaaagctgg-3' and 5'-ggggtcgacgcggccgtcagctactgtgatgccgaaca-3', designed based on published hPXR sequence. The PCR product was modified with EcoRI and NotI or with NotI and BamHI and cloned into the pCI-neo vector (Promega, Madison, WI).

Transient transfection and luciferase assay

Cells were seeded in the 12-well plates at about 30% confluence and transfected with PXR (PXR-HepG2) or vector (Vector-HT29 or V-HT29) using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. After 6 hours, transfected cells were treated with either Rifampicin (RIF) or DMSO for 24 hours, then collected and assayed for luciferase activity, in triplicate. For luciferase assay, cells were washed with cold PBS twice and then incubated with reporter lysis buffer (Promega, Madison, WI) for 10 minutes, then collected with the lysis buffer and centrifuged. 10% of the supernatant was taken for the luciferase assay according to the manufacture's manual.

Yeast two-hybrid assay

Yeast two-hybrid assay was performed using Yeastmaker Yeast Transformation System (Clonetech). AH109 competent cells were transformed with human liver library cDNAs and plasmid containing PXR-gal4 activation domain. Transformed yeast cells were plated on SD-Leu-Trp and SD-Leu-Trp-Ade-His 100 mm plates and incubated at 30 °C. Yeast colonies were counted at days 3 and 6. Transformation efficiency was calculated for each assay using PXR ligand binding domain as bait.

Co-immunoprecipitation

HepG2 cells were washed with PBS and homogenized in the Co-IP lysis buffer (20 nM Hepes, pH 7.4, 125 mM NaCl, 1% Triton X-100, 10 mM EGTA, 2 mM Na3VO4, 50 mM NaF, 20 mM ZnCl2, 10 mM sodium pyrophosphate, 1 mM dithiothreitol, and 1 mM phenylmethsulfonyl fluoride). 1X complete protease inhibitor mixture (Sigma) was added before use. Cells were homogenized in the same (above) lysis buffer. After centrifugation (12,000 X g in a microcentrifuge at 4 °C for 15 minutes), supernatant fractions were collected and incubated with antibodies and GammaBind Plus-Sepharose beads (Amersham Biosciences) for 2 hours at 4 °C on a rotary shaker. Corresponding isotype IgG was used as a negative control. The beads were washed 3 times, and the precipitated protein complexes were analyzed with Western blot.

Mammalian two-hybrid assay

The mammalian two-hybrid assay was performed using Checkmate Mammalian Two-Hybrid System (Promega). CV-1 cells were seeded in 12-well plates and transient transfected with pBIND-PXR, pACT-CNOT2, and pG5-luc. 12 hours after transfection, cells were treated with rifampicin (10 uM, for 48 hours), and luciferase activity was determined with Polarstar optima luminometer (BMG Laboratory).

GST pull-down assay

[^{35}S]Methionine-labeled PXR-LBD protein was generated with TNT-coupled Reticulocyte Lysate System (Promega) using the SP6 promoter-driven cDNA plasmid as the template. PCR-generated CNOT2 cDNA fragments were inserted in-frame into

pGEX-5X-s (Amersham Biosciences). The plasmids were expressed in *E. coli* (BL21), and fusion polypeptides were purified with glutathione-Sepharose 4B beads (Amersham Biociences) according to the manufacturer's instruction. 20 μg of each fusion polypeptide (estimated by comparison with bovine serum albumin in an SDS-PAGE gel with Coomassie Blue staining) was incubated with 20 μL of radiolabeled PXR in a total volume of 200 μL of binding reaction buffer (20 mM Hepes pH 7.9, 1% Triton X-100, 20 mM dithiothreitol, 0.5% bovine serum albumin and 100 mM KCl) for three hours at 4 °C. After incubation, beads were washed three times with the same buffer without bovine serum albumin. The bound proteins were eluted by boiling in the SDS-PAGE sample buffer and resolved by 12% SDS-PAGE gel electrophoresis. The signals were detected by autoradiography. The input control was 2 μL of the radioactive PXR.

Small interfering RNA

Two small interfering RNA-expressing plasmids were constructed by cloning the sequences targeting CNOT2 gene into pSilencer 5.1 plasmids according to the manual (Ambion). The targeting plasmids were created by inserting 5'-aatatgacaaattagaagaac-3' (CNOT2 siRNA #1) and 5'-aacgaacattcacattaggga-3' (CNOT2 siRNA #2). The siRNA plasmids and the scramble siRNA control were co-transfected with PXR-directed reporter plasmid pGL3-3A4-Luc (5) into PXR-HepG2 cells. The transfected cells were treated with rifampicin (10 uM, for 48 hours). Luciferase activity and CNOT2 protein expression were determined with luminometry and Western blotting, respectively.

Poly(A) tail PCR

Poly(A) tail polymerase chain reaction (PAT PCR) was performed to analyze the status of cytochrome P450 3A4 mRNA. RNA was harvested from HepG2 cells and PXR-HepG2 cells that were treated with 10 μM rifampicin or DMSO for 24 hours. cDNAs were produced by reverse transcriptase PCR kit using 1 μL oligo(dT) primers (500 μg/mL) and 1 μL poly(A) tail anchor primer (5'-gcgagctccgcggccgcgt$_{12}$-3'). PCR amplification of cDNAs using forward primer CYP3A4 specific (5'-tggatcgcctgaggtcagga-3') and poly(A) tail anchor as reverse primer produced a mixture of products with various poly(A) tail lengths. The PCR products were run on a 2% agarose gel and images were generated by KODAK Imaging Station.

Western blot

Western blot was performed to confirm the expression of PXR in transfected culture cells; as well as E2F1 and Rb expression in xenograft tumors. Cell lysates or homogenized tissue samples were boiled in a SDS loading buffer at 95 °C for 10 minutes. Proteins were separated by SDS-PAGE with 10% or 8% polyacrylamide gel and then transferred to the trans-blot transmembrane (Bio-Rad, Hercules, CA). Membranes were blocked in 5% milk TBST buffer (Tris buffered saline with 0.1% Tween 20) overnight at room temperature. Immunoblotting was performed by exposing membrane to primary antibodies, PXR monoclonal antibody (R&D Systems, Minneapolis, MN) at dilution of 1:1000, PXR polyclonal antibody (Santa Cruz, CA) at dilution of 1:500 or Rb monoclonal antibody (Calbiochem, Gibbstown, NJ) at dilution of 1:1000, E2F1 monoclonal antibody (sc-251, Santa Cruz Biotechnology, Santa Cruz, CA)

at dilution of 1:1000, CNOT2 monoclonal antibody (antibody produced by Tian lab) at dilution 1:500 for over 2 hours and to corresponding secondary alkaline phosphatase (AP) conjugated antibodies (1:2000) for 2 hours. The presence of the respective protein was exposed by adding Western blot AP substrate (Promega, Madison, WI) according to the manual until stopping the reaction in cold tap water.

Soft agar colony formation assay

HT-29 cells with transfected vector or PXR were mixed with culture medium containing 0.25% agarose and plated at a density of 2×10^4 per well on the pre-solidified bottom layers of the same medium containing 0.5% agarose in 6 Petri dishes (60-mm). Cells were incubated at 37 °C. After 1 week 1 ml culture medium was added upon the solidified top layer. Photos were taken of all the Petri dishes after 3 weeks of incubation. The number and size of colonies with a diameter above 100 μm were determined by ImageJ program (NIH). The results were expressed as mean±SEM.

Flow cytometry

Cells transfected with PXR or vector were cultured up to 70% confluency, treated with DMSO or rifampicin for 24 hours and harvested by trypsinization. Cells were fixed in 70% ethanol for 2 hours, followed by washing with PBS twice and resuspended in Propidium iodide (20 μg/ml) staining solution containing 1 mg/ml RNase in 10 mM PBS (pH 7.4) for 30 minutes at room temperature. Flow cytometry analysis was performed immediately in an FACS Calibur flow cytometer (Becton Dickinson) with an excitation at 488 nm and an emission at 620 nm. Triplicate experiments were performed.

In vivo tumorgenesis assay

Twenty-four female BALB/c nude mice, age 6 to 8 weeks, were divided into four groups. 7.5×10^6 HT-29 cells with transfected PXR gene or vector were subcutaneously injected into the nude mice at right flank, respectively. All animals received either corn oil or rifampicin (100 mg/kg/day intraperitoneal) treatment from day 6 to 16. The tumor size was measured by a vernier caliper every two days from day 6 to 16 after cell implantation. The volume was calculated by a formula: $V=0.5a \times b^2$, where "a" is the long diameter and "b" is the short diameter. The animals were sacrificed at day 16. The tumor was removed, weighed and cut into two pieces. One piece was fixed with 4% neutral buffered formaldehyde solution and another one was frozen in liquid nitrogen for further assays.

Immuno-cytochemistry

To confirm the expression of transfected PXR *in situ*, immuno-cytochemical staining with PXR antibody was performed in cultured cells. PXR-HT29 and Vector-HT29 cells were seeded in the 8-well chamber slides, cultured for two days and fixed with 4% neutral buffered formaldehyde solution for 20 minutes. Microwave heated antigen retrieval was performed in 0.01 mol/L citric acid buffer (pH 6.0) for 15 minutes. Cells were treated with 0.1% Triton X-100 for 10 minutes, then blocked with normal donkey serum for 30 minutes and then incubated with PXR mouse monoclonal antibody (R&D Systems, Minneapolis, MN) at dilution 1:100 overnight at 4°C. After washing with phosphate buffered saline containing 0.1% tween-20 (PBST), the biotinylated secondary antibody and the streptavidin-biotin complex (Invitrogen, Carlsbad, CA) were

applied, each for 30 minutes at room temperature with an interval PBST washing. 3,3'-diaminobenzidine (Sigma, St. Louis, MO) solution (0.4 mg/ml, with 0.003% hydrogen peroxide) was used as a substrate for developing color. The slides were then counterstained with hematoxylin, dehydrated and mounted with coverslips. The results were visualized on an Olympus (AH-3, Olympus, Japan) microscope equipped with a SPOT INSIGHT COLOR digital camera, and images were obtained using SPOT DIGITAL CAMERA SYSTEMS software (Diagnostic Instruments, Inc., USA).

Immuno-histochemistry

Immuno-histochemical staining was performed in HT-29 tumors from nude mice. Paraffin-embedded sections were deparaffinized, rehydrated, and microwave heated for 15 minutes in 0.01 mol/L citric buffer (pH 6.0) for antigen retrieval. Then, 3% hydrogen peroxide was applied to block endogenous peroxidase activity. After 15 minutes of blocking with normal serum (invitrogen, Carlsbad, CA), the primary antibody or corresponding control isotype IgG were applied and incubated overnight at 4°C. Slides were washed thrice with PBS, each for 5 minutes. The biotinylated secondary antibody and the streptavidin-biotin complex were applied, each for 30 minutes at room. After rinsing with PBS, the slides were immersed for 10 minutes in 3,3'-diaminobenzidine (Sigma, St. Louis, MO) solution (0.4 mg/mL, with 0.003% hydrogen peroxide), monitored under microscope and stop the reaction with distilled water, counterstained with hematoxylin, dehydrated, and coverslipped. The primary antibodies used are as follows: ki-67 (sc-15402, Santa Cruz Biotechnology, Santa Cruz, CA), PXR

(PP-H4417-00, R&D Systems, Minneapolis, MN) and E2F1 (sc-251, Santa Cruz Biotechnology, Santa Cruz, CA).

Immuno-fluorescence

Paraffin-embedded sections were deparaffinized, rehydrated, and microwave heated for 15 minutes in 0.01 mol/L citric buffer (pH 6.0) for antigen retrieval. After a blocking with 10% donkey serum (Jackson ImmunoResearch, West Grove, PA) for 30 minutes, the primary antibody solution containing mouse anti-PXR antibody (1:100) and rabbit anti-Ki-67 antibody (1: 100), or mouse anti-CNOT2 (1:250) and rabbit anti-PXR (1:500) or anti-coilin (1:500) or the solution of corresponding isotype control IgGs at the same concentration with primary antibody were applied and incubated overnight at 4°C. Sections were washed with PBS for 3 times, each for 5 minutes. The secondary antibody solution containing donkey anti-mouse antibody conjugated with orange-red fluorescent AF568 and donkey anti-rabbit antibody conjugated with green fluorescent AF488 (both from invitrogen, Carlsbad, CA and at dilution of 1:1000) was applied in dark for 30 minutes. Slides were washed 3 times with PBS and mounted with aquatic medium contained DAPI. The primary antibodies used are as follows: anti-PXR from R&D Systems (Minneapolis, MN), anti-Ki-67 from Santa Cruz Biotechnologies (Santa Cruz, CA),anti-coilin from BD Biosciences (San Jose, CA) and mouse anti-CNOT2 which our lab produced.

TUNEL staining

Terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) staining was done using the *In situ* Cell Death Detection kit (Roche Applied Science,

Indianapolis, IN) following the instructions of the manufacturer. Briefly, 4-μm-thick formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by hydrogen peroxide and tissue protein was hydrolyzed with proteinase K. Positive control are sections treated with DNase I 1,000 units/mL. Negative control sections are incubated with label solution (without terminal deoxynucleotidyl transferase enzyme). All other sections were incubated with TUNEL reaction mixture (fluorescein-labeled nucleotides) at 37°C for 1 hour in a humid chamber, incubated with converter-POD solution (antifluorescein antibody conjugated with POD) for 30 minutes at 37°C, treated with DAB, and counterstained with hematoxylin.

Quantitative measurements

The quantitative analysis of immuno-histochemical staining with nuclear positive (Ki-67, TUNEL, Rb and E2F1) was performed by PhotoShop and ImageJ (NIH) programs. 10 to 15 photos per sample were taken randomly. Briefly choose the positive staining by PhotoShop Color Range and save it as the criterion for all samples. Fill the chosen area with white color and fill the rest area with black color. Then the function of Analyze Particle in ImageJ Program was applied to count the number of positive nuclei.

Statistics

All data were analyzed by comparing means with One-Way ANOVA method and followed an additional Duncan Post Hoc test for the results of luciferase activity and cell cycle assay using SPSS (Version 11.5.0). Data was shown as mean ± SEM and $P < 0.05$ denotes the presence of a statistically significant difference.

RESULTS

Substudy 1: Pregnane X receptor interacts with CNOT2 of the CCR4-NOT complex

Identification of PXR-LBD interactive partners by yeast two-hybrid assay

PXR ligand binding domain (PXR-LBD) was inserted into pGBKT7 as the bait for yeast two-hybrid assay to screen the human cDNA liver library for interactive partners. Interactions were observed between PXR and many positive clones, including known PXR partner retinoic acid receptor β (RXRβ) and metastasis associated protein 1 (MTA1). In total, over one million cDNA clones were screened with PXR-LBD as bait yielding one interactive partner of particular interest to our lab. One of the positive clones was a partial cDNA of CNOT2, amino acids 183-540. The cDNA fragment was extracted from yeast cells, inserted in pACT2 plasmid and transformed into AH109 yeast for confirmation testing with α-gal assay (Figure 7A) as well as yeast growth assay (7B). The results indicated that PXR ligand binding domain interacts with CNOT2 fragment.

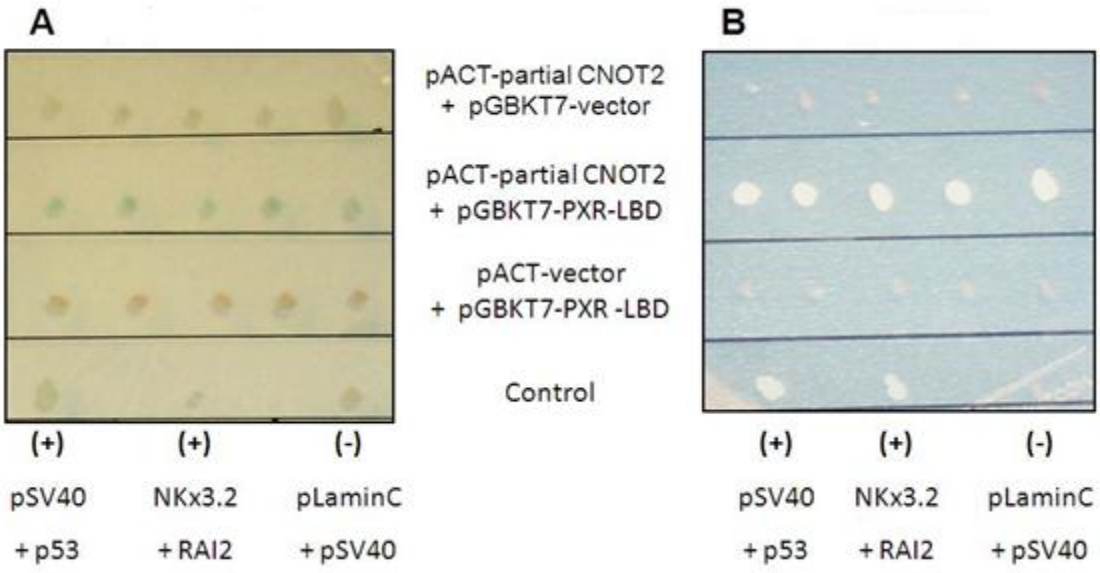

FIGURE 7: Yeast two-hybrid assay revealed that CNOT2 may be an important interactive partner of PXR. α-gal assay and yeast growth assay were conducted to verify the initial results from the yeast two-hybrid assay. AH109 yeast colonies were plated onto SD-2 and SD-4 (with x-gal) 100 mm Petri dishes. The plates were incubated at 30 °C for 3 days, then photographed.

To characterize the interactive domains between PXR-LBD and CNOT2, PXR-LBD was inserted into pGBKT7 (bait vector). CNOT2 fragments corresponding to different domains were cloned into pACTs plasmid (prey vector). [Figure 8]

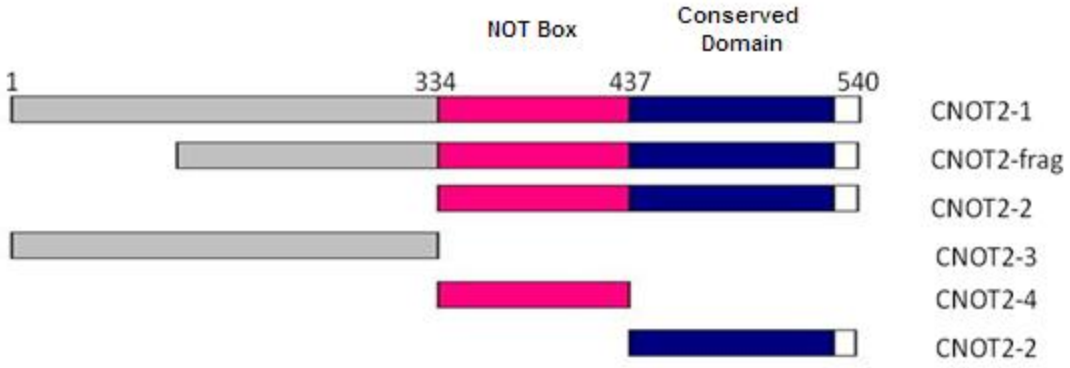

FIGURE 8: Engineered CNOT2 fragments used in yeast two-hybrid and GST pull down assays.

The yeast were cotransformed with PXR-LBD bait and various fragments of CNOT2. The transformed yeast were plated on SD –Leu-Trp-X-α-gal media for α-gal assay (Figure 9A) as well as onto SD –Leu-Trp-Ade-His media for growth assay (9B). The conserved domain and the NOT Box domain interacted with PXR-LBD in yeast two-hybrid assay and growth assay. The other CNOT2 fragments showed little interaction with the PXR ligand binding domain. The NOT Box domain alone showed a greater interaction with PXR-LBD; but there was a synergistic interaction with both the conserved domain and the NOT Box.

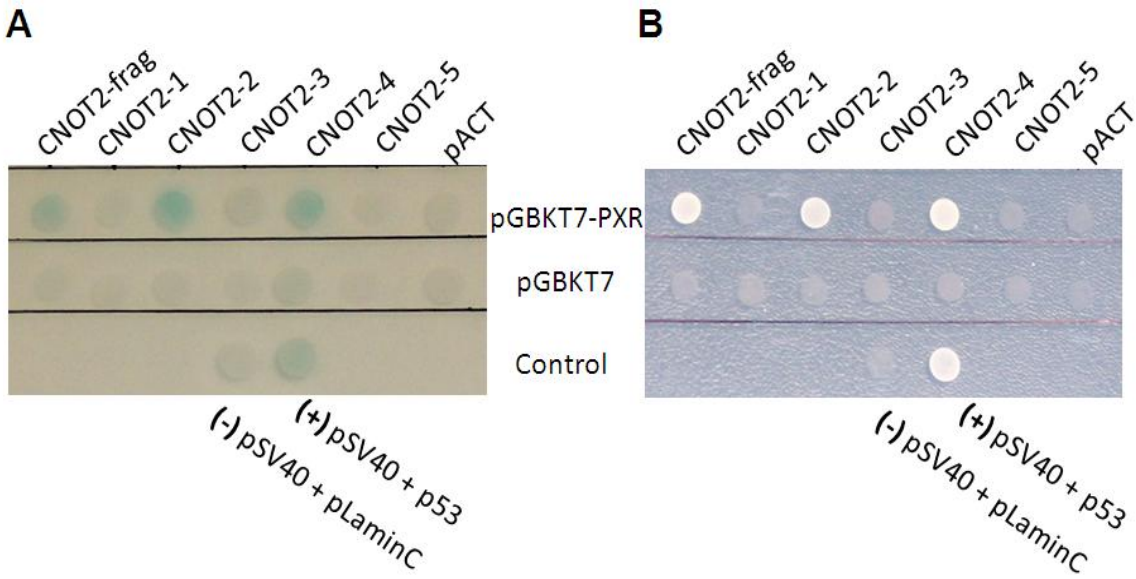

FIGURE 9: PXR ligand binding domain interacts with specific domains of CNOT2. α-gal assay and growth assays were performed with various fragments of CNOT2. CNOT2 fragments were created by inserting into pACT vector. Plasmid interaction pSV40 with p53 was used as the positive control and pSV40 with pLaminC was used as the negative control. (Results were generated in collaboration with Liu Duan.)

Mapping of CNOT2 domains involved in the interaction with PXR-LBD

CNOT2 gene fragments (configured as in Figure 8) were fused to GST gene in order to produce GST-fusion peptides. The fusion peptides were expressed in *E.coli* and conjugated to glutathione beads. The GST pull-down protein complexes were separated by SDS-PAGE; and radioactive signals of 35S-labeled PXR and luciferase (negative control) were recorded by autoradiography (Figure 10A) and Coomassie blue stain (10B). Results from the GST pull-down confirm that PXR-LBD and CNOT conserved and NOT Box domains interact directly.

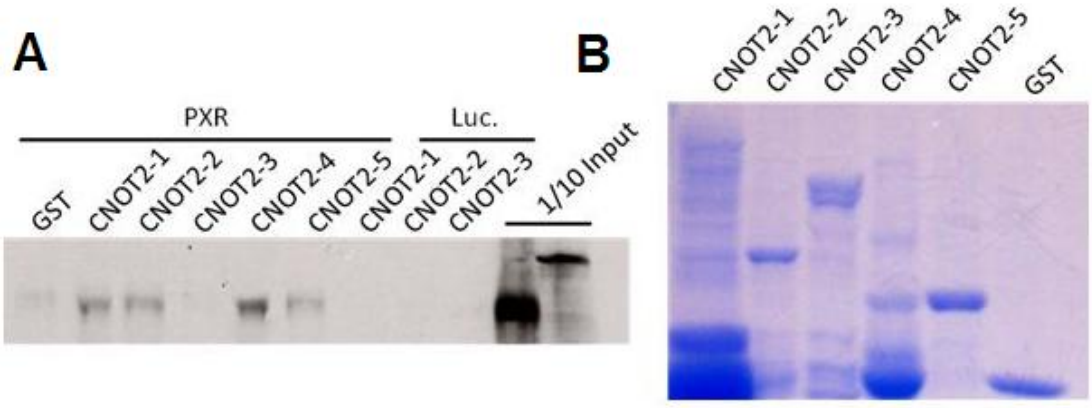

FIGURE 10: GST pull down confirms that PXR and CNOT2 directly interact. [^{35}S]Methionine-labeled PXR-LBD protein was generated using the SP6 promoter-driven cDNA plasmid as the template. PCR-generated CNOT2 cDNA fragments were inserted in-frame into pGEX-5X-s. The plasmids were expressed in *E. coli* (BL21), and fusion polypeptides were purified with glutathione-Sepharose 4B beads. 20 µg of each fusion polypeptide (estimated by comparison with bovine serum albumin in an SDS-PAGE gel with Coomassie Blue staining) was incubated with 20 µL of radiolabeled PXR in a total volume of 200 µL of binding reaction buffer for 3 hours at 4 °C. After incubation, beads were washed three times. The bound proteins were eluted by boiling in the SDS-PAGE sample buffer and resolved by 12% SDS-PAGE gel electrophoresis (A). The signals were detected by autoradiography (B). The input control was 2 µL of the radioactive PXR. (Results were generated in collaboration with Liu Duan.)

PXR and CNOT2 interact directly in mammalian cell lines

PXR-LBD was fused with Gal4 DNA binding domain (DBD) in plasmid pBind (Gal4-PXR). CNOT2 was fused to activation domain (VP16) in plasmid pACT (Promega). Luciferase reporter gene driven by gal4 DNA binding consensus sequences was transiently co-transfected with Gal4-PXR and pACT-CNOT2 into CV-1 cells. The cells were treated with 5 µM rifampicin (RIF) 6 hours after transfection. Luciferase activity was measured 24 hours after transfection (Figure 11A). The results demonstrated that PXR and CNOT2 interact within CV-1 cells in a PXR ligand-

dependent manner. There was no induction of luciferase activity without transfection of both Gal4-PXR and pACT-CNOT2.

The interaction between CNOT2 and PXR was determined by co-immunoprecipitation. Cos7 cells were transfected with HA-tagged CNOT2 and Flag-tagged PXR plasmids. Cells were treated with 10 μM RIF or DMSO for twenty four hours after transfection. Cell lysate was precipitated by beads conjugated with HA or Flag antibody. Western blot was used to detect anti-Flag antibody (Figure 11B). 10% of the total lysate was loaded as the input. There was an increase in precipitated protein when treated with PXR ligand RIF. The results indicate that Flag-PXR was successfully precipitated with HA-tagged CNOT2 protein.

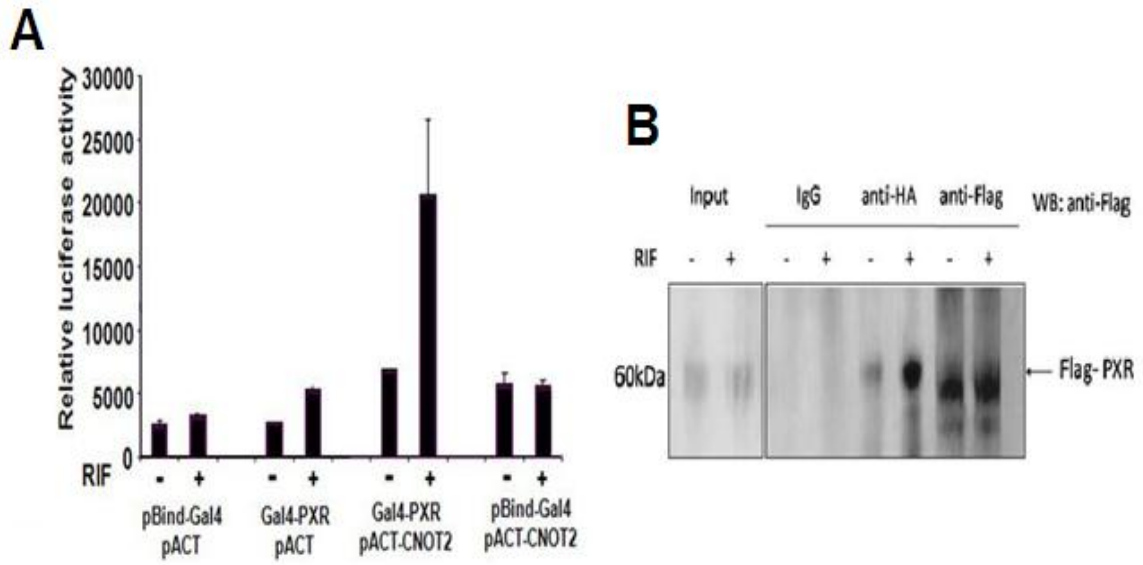

FIGURE 11: Mammalian two-hybrid and co-immunoprecipitation assays indicate that PXR and CNOT2 directly interact within mammalian cell lines**.** For the mammalian two-hybrid assay CV-1 cells were seeded in 12-well plates and transient transfected with pBIND-PXR, pACT-CNOT2, and pG5-luc. 12 hours after transfection, cells were treated with rifampicin (10 uM, for 48 hours), and luciferase activity was determined (A). For the co-immunoprecipitaion assay HepG2 cells were co-transfected with Flag-PXR and HA-CNOT2 plasmids. Cells were treated with 10 μM RIF for 48 hours before harvest. Transfected HepG2 cells were homogenized in lysis buffer, centrifuged to collect proteins and supernatant fractions were collected and incubated with antibodies and GammaBind Plus-Sepharose beads for 2 hours at 4 °C on a rotary shaker. Corresponding isotype IgG was used as a negative control. The beads were washed 3 times, and the precipitated protein complexes were analyzed with Western blot (B) using an antibody against Flag-PXR. 10% of the supernatant collected before incubation with beads was used for the input. (Results were generated in collaboration with Liu Duan.)

The interaction between CNOT2 and PXR suggests the CCR4-NOT complex regulates PXR-transcriptional activity

The effect of CNOT2 on the transcriptional activity of PXR was tested in HepG2 cells. HepG2 cells with constitutively expressed PXR were cotransfected with

PXR-driven luciferase reporter gene and pTargeT-CNOT2, mammalian expression vector under control of the CVM promoter. Cells were treated with rifampicin for 48 hours before proteins were harvested for the luciferase assay. Relative luciferase activity indicates that PXR-regulated gene expression is suppressed when CNOT2 and PXR interact (Figure 12A). Relative luciferase activity was markedly decreased with CNOT2 expression ($p<0.01$).

PXR-HepG2 cells were transiently co-transfected with PXR-driven luciferase reporter gene, CNOT2 siRNA and scramble RNA as the negative control. Two siRNA sequences (CNOT2 siRNA #1 and CNOT2 siRNA #2) of 0.5 μg each were used for the knockdown. (Figure 12B) PXR was activated by treatment of 10 μM rifampicin and luciferase activity was measured 48 hours after transfection. We continued to use CNOT2 siRNA #1 sequence since it had the greatest silencing effect and 0.5 μg and 1.0 μg of RNA were used to measure the knockdown effects (Figure 1C). siRNA CNOT2 knockdown results in enhancement of PXR-transcriptional activity as indicated in PXR-driven luciferase activity assay.

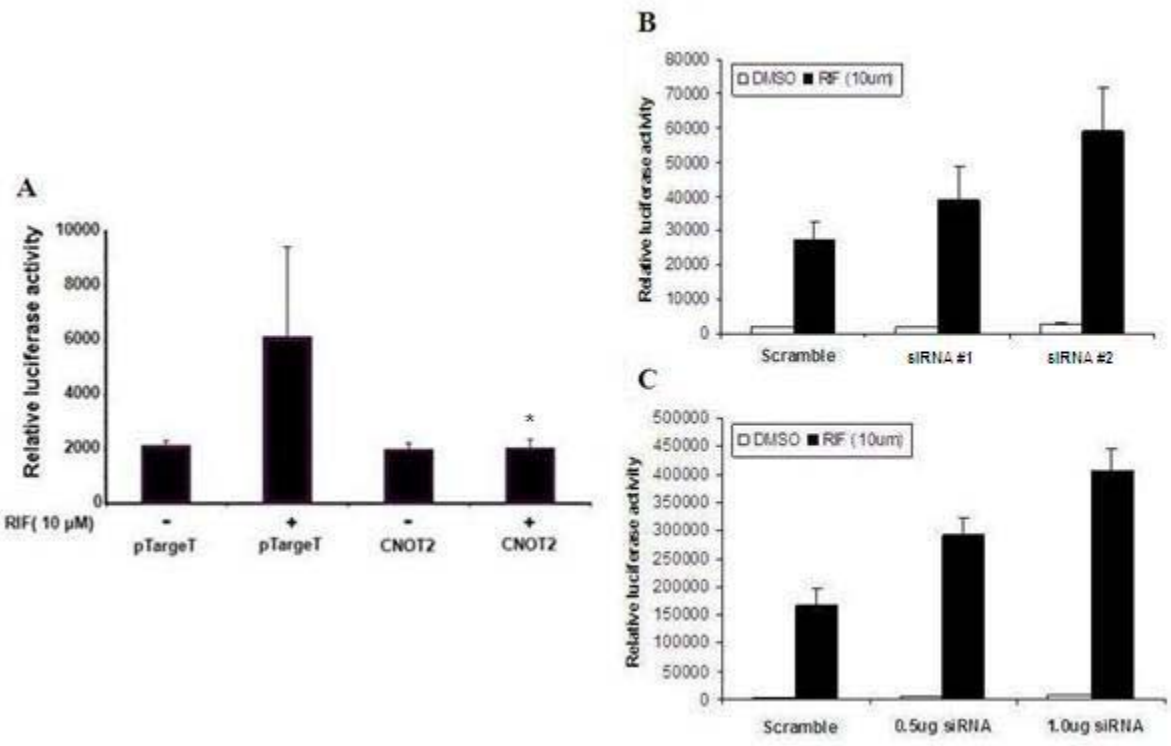

FIGURE 12: Transcriptional activity of PXR is suppressed by PXR-CNOT2 interaction. PXR-HepG2 cells were cotransfected with PXR-driven luciferase reporter gene and pTargeT-CNOT2, then treated with rifampicin for 48 hours. For luciferase assay, cells were washed with PBS and incubated with reporter lysis buffer for 10 minutes, then collected with the lysis buffer and centrifuged to collect proteins. 10% of the supernatant was taken for the luciferase assay. siRNA knockdown of CNOT2 was performed to determine the effect on PXR-transcriptional activity. Two small interfering RNA-expressing plasmids (CNOT2 siRNA #1 and CNOT2 siRNA #2) were constructed by cloning the sequences targeting CNOT2 gene into pSilencer 5.1 plasmids. The siRNA plasmids and the scramble siRNA control were co-transfected with PXR-directed reporter plasmid pGL3-3A4-Luc into PXR-HepG2 cells. The transfected cells were treated with rifampicin (10 uM, for 48 hours). CONT2 siRNA #1 sequence had the greatest effect and was thus used for subsequent assays. Luciferase activity was determined with luminometry (B and C). The results were displayed as the folds of control group. (Results were generated in collaboration with Ke Sui.)

CYP3A4 RNA turnover is affected by PXR-CNOT2 interaction

To analyze mRNA turnover activity and mRNA stability PAT PCR was performed. The diagram shows that a gene specific primer anneals to a set location and a poly(A) tail anchor primer can anneal anywhere on the length of repeated adenine residues (Figure 13A). The PAT PCR results indicate when there is co-transfection of CNOT2 and PXR, deadenylation of the poly(A)tail is less (Figure 13B). The smear seen in lanes containing PXR indicate there were many different lengths of PCR product; whereas the lanes without PXR have mostly the same length of product and therefore do not show a smear. There was little effect after treatment with the PXR ligand rifampicin (RIF).

Cellular localization indicates PXR and CNOT2 interact within the nucleus of HepG2 cells

To determine the location of PXR and CNOT2 protein immuno-fluorescent double staining was performed in HepG2 cells. The cells were treated with rifampicin (RIF) or DMSO for 24 hours. The upper panel of images is from HepG2 cells without PXR expression; the lower panel of images has PXR expression. The antibody against CNOT2 indicates that when PXR is over-expressed CNOT2 protein relocates closer to the nucleus (compare Figures 14A/C to 14E/G). No apparent differences were observed in cells treated with RIF.

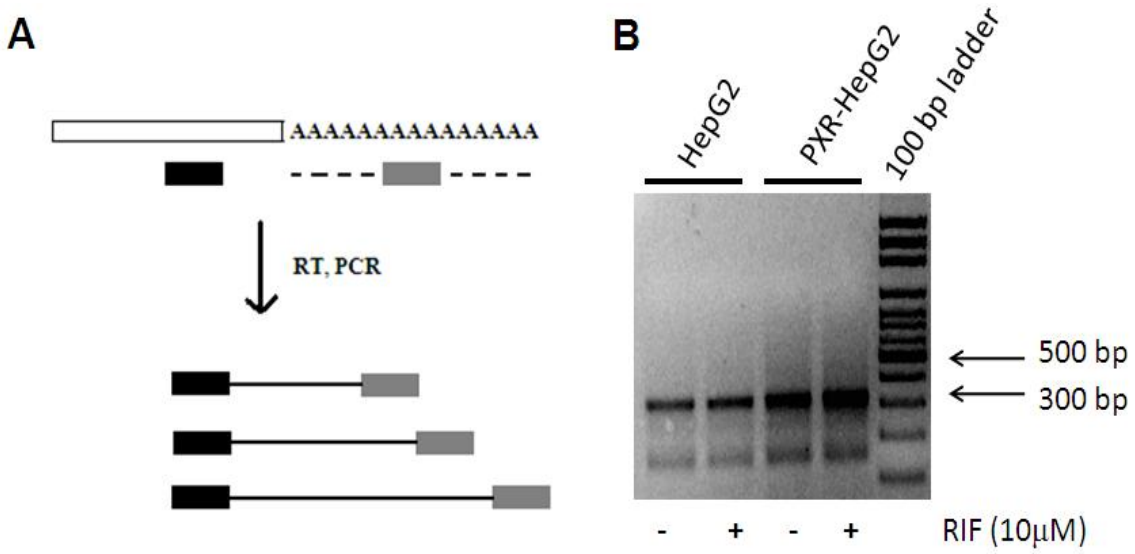

FIGURE 13: Results indicate that PXR over expression prevent mRNA transcript degradation through poly(A) tail deadenylation. The schematic of poly(A) tail PCR (PAT PCR) shows that mRNA transcripts with variable poly(A) tail lengths were harvested from HepG2 or PXR-HepG2 cells. . Total RNA was harvested from HepG2 cells and PXR-HepG2 cells that had been treated with 10 μM rifampicin or DMSO for 24 hours. cDNAs were produced by reverse transcriptase PCR using oligo(dT) forward primer and reverse primer that anchors to the poly(A) tail. PCR amplification of cDNAs using a CYP3A4 specific forward primer and poly(A) tail anchor as reverse primer produced a mixture of products with various poly(A) tail lengths. The CYP3A4 target product with extended poly(A) tail is approximately 360 bp. The PCR products were analyzed by gel electrophoresis on a 2% agarose gel and images were generated by KODAK Imaging Station (B).

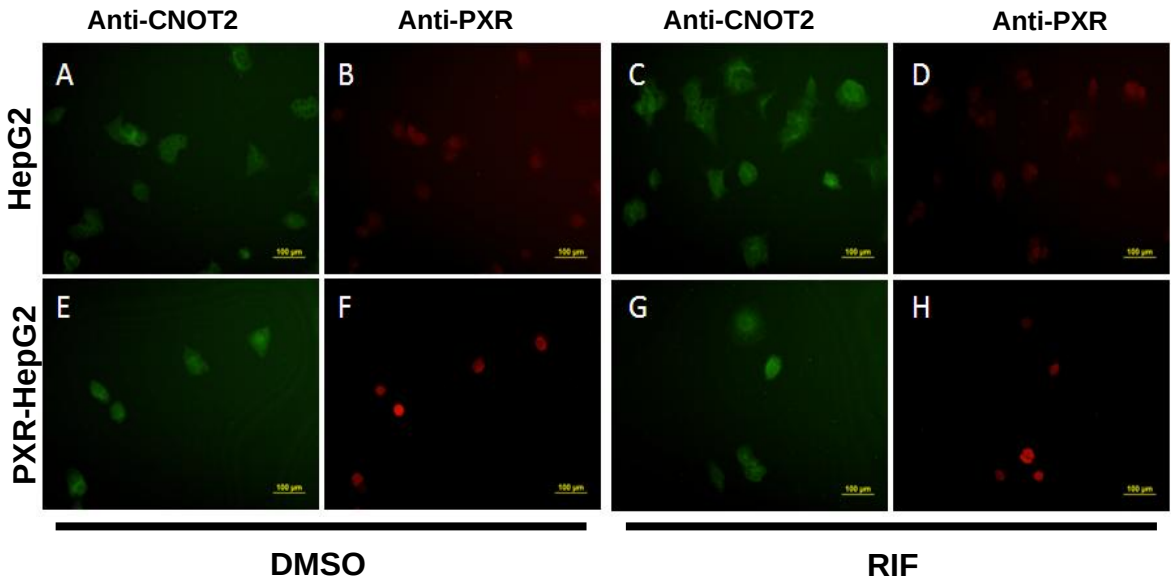

FIGURE 14: Immuno-fluroscent microscopy of HepG2 and PXR-HepG2 cells indicates that PXR and CNOT2 proteins may colocalizes within the nucleus. HepG2 and PXR-HepG2 cells were treated with 10 µM rifampicin or DMSO for 24 hours. Cells were washed with PBS and fixed with 4% parafomaldehyde. Monoclonal antibody against CNOT2 (1:500) and polyclonal antibody against PXR (1:1000) were applied and incubated at room temperature for 24 hours. Corresponding fluorescent secondary antibodies (anti-mouse green and anti-rabbit Alexia red) were applied and incubated at room temperature for 4 hours. DAPI mounting solution was applied and coverslips mounted.

An antibody against coilin was used to determine the presence of Cajal bodies within the nucleus. In general, we observed that there were fewer coilin foci present in HepG2 cells lacking PXR expression. (Figure 15A) The numbers of coilin foci were not altered by treatment with rifampicin. In Cos7 cells, GFP-PXR fusion protein was produced to pinpoint the precise location of PXR, instead of an over-expression vector that results in illuminating the entire nucleus (as in Figure 14F/H).Using the GFP setting on the fluorescent microscope we were able to visualize the GFP-PXR protein (Figure

15B). The cellular locations of coilin and GFP-PXR protein indicate that they might colocalize within the nucleus.

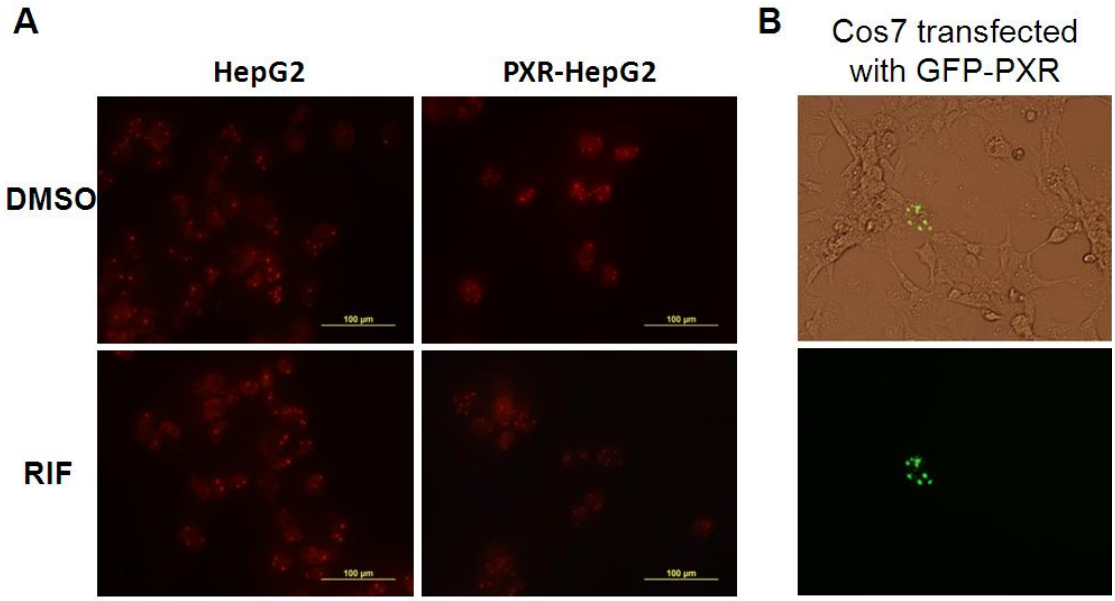

FIGURE 15: Immuno-fluorescent microscopy indicates that expression of PXR increases the number of coilin foci. HepG2 and PXR-HepG2 were treated with DMSO and 10 µM rifampicin (RIF) for 24 hours. Cells were washed with PBS and fixed with 4% paraformaldehyde. Monoclonal mouse anti-coilin (1:500) was applied and incubated for 24 hours. Cos7 cells were transfected with engineered GFP-PXR plasmid that produce PXR protein with C-terminal green fluorescent protein. The presence of GFP was detected by OLYMPUS microscope.

Substudy 2: Pregnane X receptor is involved in colon cancer progression

Restoration of PXR by stable transfection of PXR gene in HT29 cells

To investigate the effects of PXR on tumor cell growth we have restored the PXR expression through transfection of human PXR into colon cancer cell line. HT29 cells with transfected vector are PXR negative (Fig 16A) whereas cells stably transfected with PXR exhibit over expressed PXR protein which is localized in the nucleus (Figure

16B) as determined by immuno-cytochemical staining. [Image to scale at 50 μm] The PXR regulated response was tested by co-transfection with PXR and its driven luciferase reporter gene. The relative luciferase activity was markedly increased 4-fold ($p<0.01$) after treatment with PXR ligand rifampicin (RIF) in PXR transfected cells (Fig 16C).

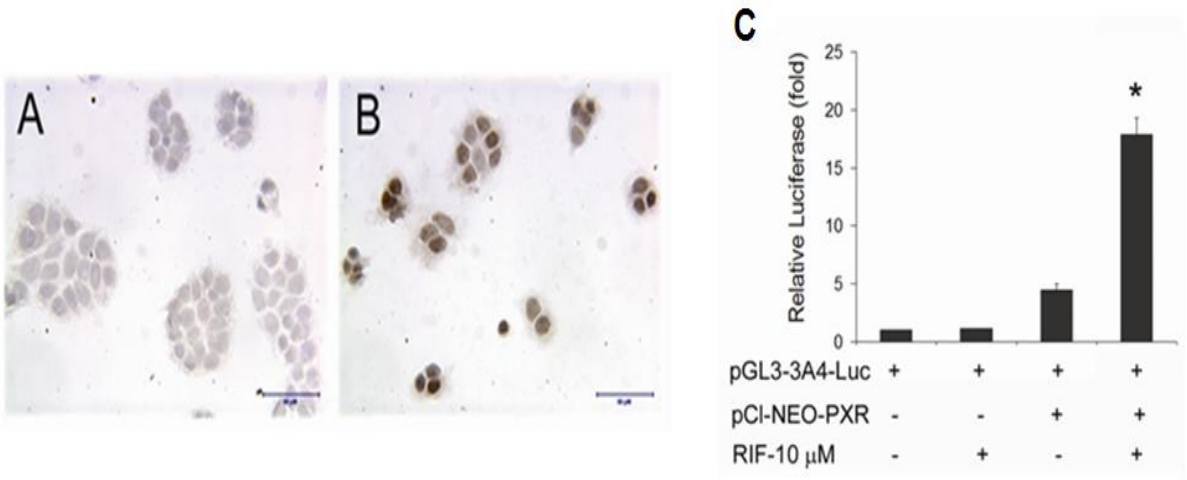

FIGURE 16: Stabilization of PXR-HT29 cell line. To confirm the expression of transfected PXR *in situ*, immuno-cytochemical staining with PXR antibody was performed in cultured cells. PXR-HT29 and Vector-HT29 cells were seeded in the 8-well chamber slides, cultured for two days and fixed with 4% neutral buffered formaldehyde solution for 20 minutes. Cells were blocked with normal donkey serum for 30 minutes and then incubated with PXR mouse monoclonal antibody (at dilution 1:100) overnight at 4°C. After washing, the biotinylated secondary antibody and the streptavidin-biotin complex were applied, each for 30 minutes at room temperature. 3,3'-diaminobenzidine solution was used as the substrate for developing color. The slides were then counterstained with hematoxylin, dehydrated and mounted with coverslips. The results were visualized on an Olympus microscope equipped with a SPOT INSIGHT COLOR digital camera (A and B). Luciferase assay was conducted to measure PXR-transcriptional activity to determine if the PXR transfected gene is functioning normally. HT29 cells were transfected or cotransfected with pGL3-3A4-luc reporter gene and/or pCI-NEO-PXR, respectively. Cells were treated with rifampicin (10 μM for 48 hours) before proteins were harvested for the luciferase activity assay. (Results were generated in collaboration with Nengtai Ouyang.)

PXR inhibited cell proliferation and anchorage-independent colony formation of HT29 cells

Upon expression of PXR through stable transfection, we noted cells transfected with PXR grew slower as compared to Vector-HT29 cells. To further characterize the tumor suppressive effect of PXR, we performed anchorage-independent growth assay in soft agar culture (Figure 17B and 17C). The presence of PXR in these cells significantly inhibited colony formation by 34% (399.7±26.7 vs. 264.0±21.1, $p<0.01$, Figure 17A) after 3 weeks in culture. Treatment with the PXR ligand rifampicin did not change cell growth and colony formation as compared to the DMSO control (data not shown).

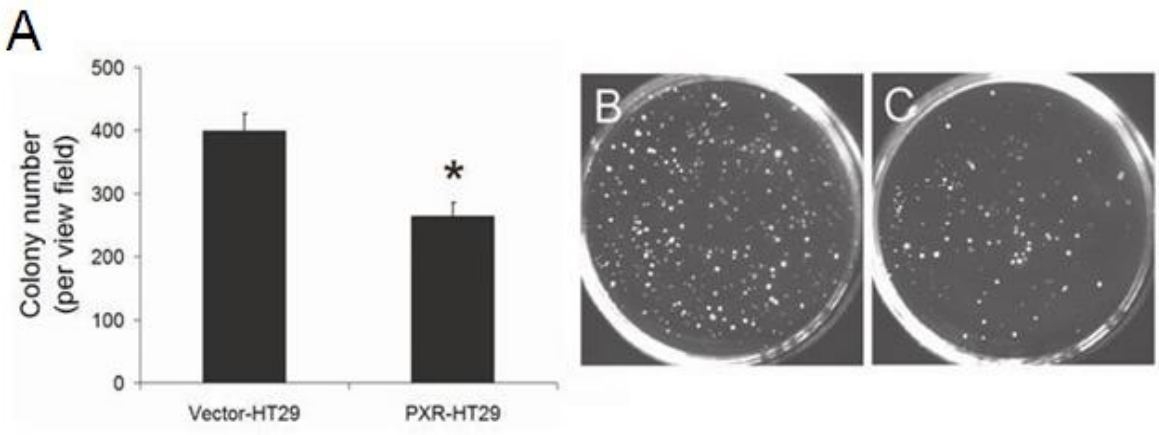

FIGURE 17: Soft agar colony formation assay indicates that the number of cells is less in PXR-HT29. HT-29 cells with transfected vector or PXR were mixed with culture medium containing 0.25% agarose and plated on 60 mm Petri dishes containing 0.5% agarose. Cells were incubated at 37 °C. All dishes were photographed after 3 weeks. The number and size of colonies with a diameter above 100 μm were determined by ImageJ program (NIH). The results were expressed as mean±SEM. (Soft agar assay conducted by Ke Sui)

Restoration of PXR expression inhibited tumor growth *in vivo*

To further analyze the growth inhibitory effects of PXR, Vector-HT29 and PXR-HT29 cells were injected subcutaneously into nude mice. The tumors that formed were removed at 17 days (Figure 18A). The tumor volume calculated by both long diameter and short diameter in PXR-HT29 group was significantly smaller than that in Vector-HT29 group (Corn oil treated: 173 mm^3 ± 18 vs. 441 mm^3 ± 78, $p<0.05$; RIF treated: 164 mm^3 ± 18 vs. 396 mm^3 ± 84, $p<0.05$) at day 16 (Figure 18B). The tumors were removed from mice upon the termination of the experiments. Figure 3B shows representative tumors from vehicle treated animals. The final tumor weight shows a significant difference between Vector-HT29 and PXR-HT29 groups (Corn oil treated: 485 mg ± 69 vs. 223 mg ± 37, $p<0.01$; RIF treated: 471 mg ± 73 vs. 190 mg ± 32, Figure 18C) but RIF treatment did not produce any effect.

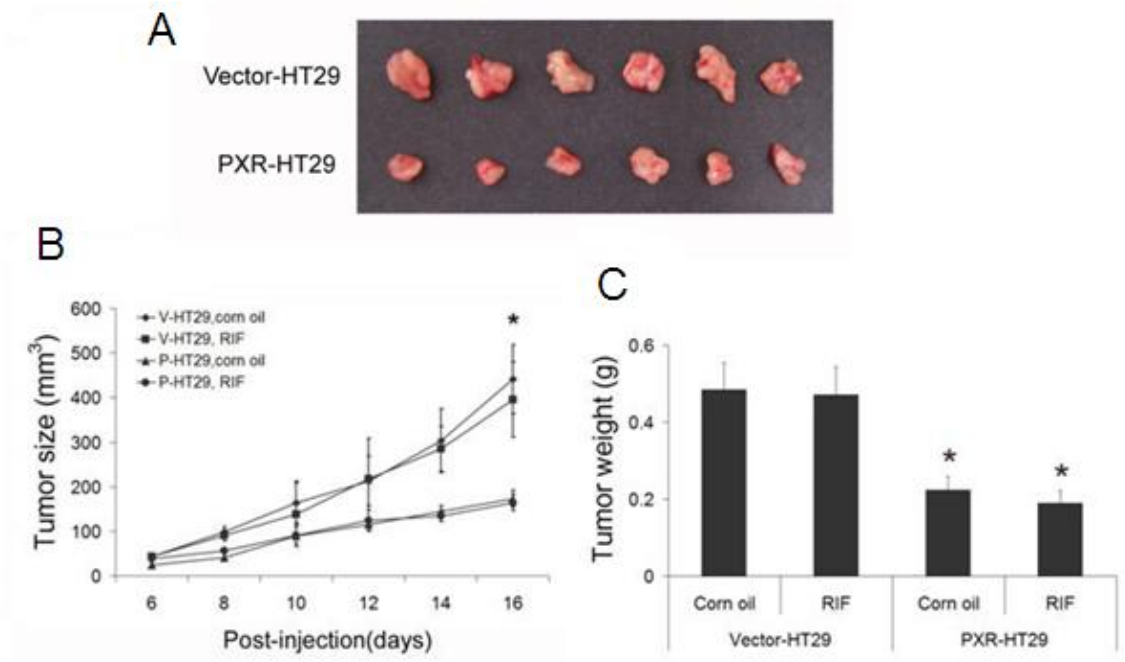

FIGURE 18: *In vivo* tumorgenesis model indicates growth is inhibited in tumors with PXR expression. Twenty-four female BALB/c nude mice, age 6 to 8 weeks, were divided into four groups. 7.5×10^6 HT-29 cells with transfected PXR gene or vector were subcutaneously injected into the nude mice at right flank. All animals received either corn oil or rifampicin (100 mg/kg/day intraperitoneal) treatment from day 6 to 16. The tumor size was measured by a vernier caliper every two days from day 6 to 16 after cell implantation; the volume was calculated. The animals were sacrificed at day 16, tumors removed, weighed and preserved. (Results were generated in collaboration with Nengtai Ouyang, Ke Sui, Ying Xie and Hongmei Cui.)

Immuno-histological analysis of suppression of proliferation by PXR in xenograft tumor tissues

To determine the mechanism of PXR-dependent inhibition of tumor growth in nude mice, we first measured cellular proliferation and apoptosis by immuno-histochemistry, immuno-fluorescent double staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in these tumor tissues (corn oil treated tumors only). Over expression of nuclear PXR protein is retained in most tumor

cells from the PXR-HT29 xenografts but not in Vector-HT29 tumors (Figure 19A and 19B). The cell proliferating marker Ki-67 is positive in the nucleus of cancer cells (Figure 19C and 19D). The number of Ki-67 positive cells per field (a photo at 400× magnification) is significantly lower in PXR-HT29 group than in the Vector-HT29 control group (50.13±4.96 vs. 30.47±5.19, $p<0.01$, Figure 19H). The TUNEL staining shows positive with brown color in the scattered single cells and apoptotic bodies (Figure 19E and 19F) in all tumor samples. The positive cells were counted by ImageJ program; however, there was no statistically significant differences in the number of apoptotic cells between these two groups (5.79±0.57 vs. 5.03±0.53, $p>0.05$, Figure 19I). Furthermore, the immuno-fluorescent double staining with Ki67 and PXR showed a mutually exclusive distribution pattern (Figure 19G).

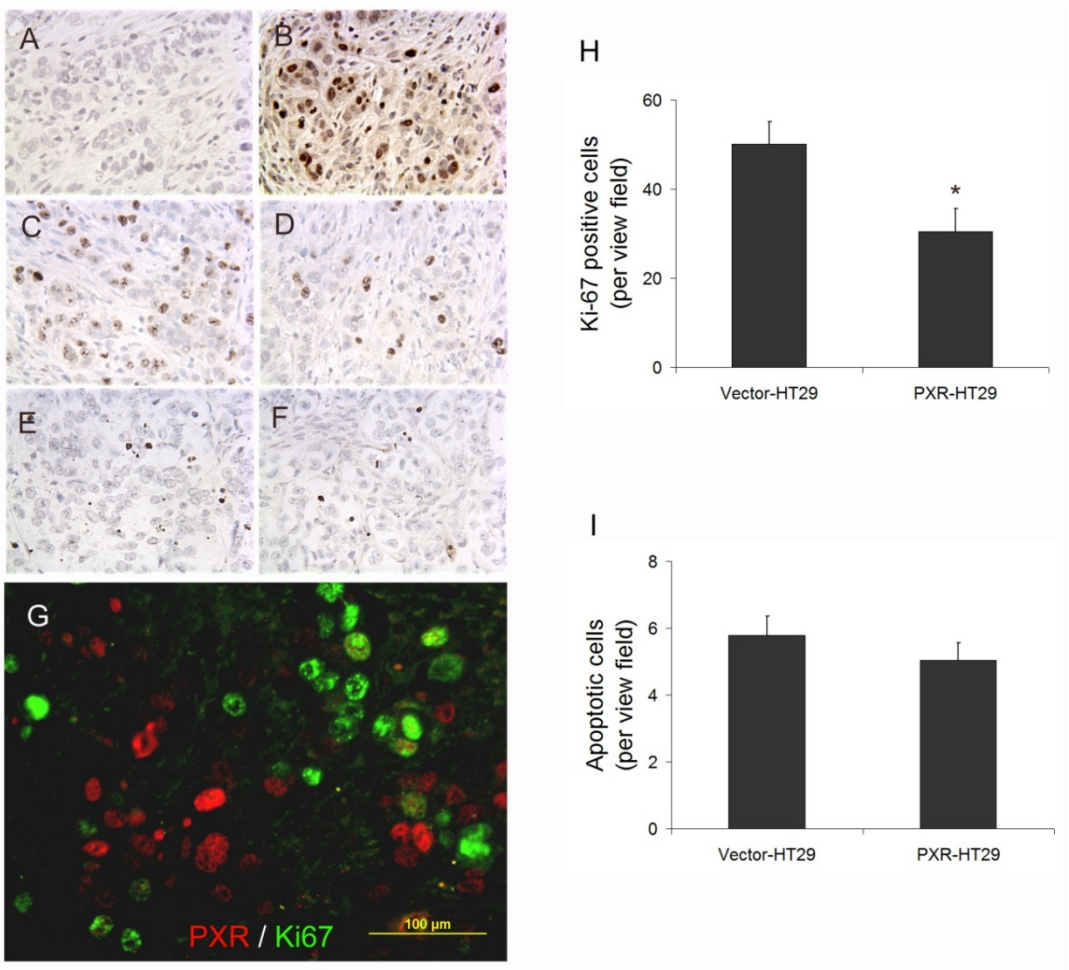

FIGURE 19: Analysis of xenograft tumor tissues indicates cells are less proliferative in PXR positive samples but apoptosis is not affected. Terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) staining was performed on HT29 and PXR-HT29 cells (E and F). 4-µm-thick formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated. Positive controls are sections treated with DNase I 1,000 units/mL. Negative control sections are incubated with label solution (without terminal deoxynucleotidyl transferase enzyme). All other sections were incubated with TUNEL reaction mixture (fluorescein-labeled nucleotides) at 37°C for 1 hour, incubated with converter-POD solution (antifluorescein antibody conjugated with POD) for 30 minutes at 37°C, treated with DAB, and counterstained with hematoxylin. Cells stained positive for apoptosis are dark colored. For immuno-fluroescence double staining (Figure 19G)HT29 and PXR-HT29 cells were blocked with 10% donkey serum for 30 minutes, the primary antibody solution containing mouse anti-PXR antibody (1:100) and rabbit anti-Ki-67 antibody (1: 100) or the solution of corresponding isotype control IgGs at the same concentration with primary antibody were applied and incubated overnight at 4°C. Sections were washed with PBS for 3 times, each for 5 minutes. The secondary antibody solution containing donkey anti-mouse antibody conjugated with orange-red fluorescent AF568 and donkey anti-rabbit antibody conjugated with green fluorescent AF488 was applied in dark for 30 minutes. Slides were mounted with aquatic medium contained DAPI. (Immuno-histochemistry conducted by Ouyang Nengtai)

PXR expression in colon cancer cells leads to G_0/G_1 cell cycle arrest

To explore the mechanism of the growth suppressive effects of PXR in HT29 cells, a cell cycle analysis was performed by staining with propidium iodide and performing flow cytometry. Arrest of cell cycle progression contributes to the inhibition of tumor cell growth. The results showed that the percentage of cells at G0/G1 phase is significantly higher in PXR-HT29 cells than in Vector-HT29 cells (67.2%±1.9 vs. 40.5%±1.4, $p<0.01$) and the percentage of the cell population at S phase and G2/M phase is significantly lower in PXR-HT29 cells than in Vector-HT29 cells, (25.2%±0.6 vs. 43.6%±1.2, $P<0.01$, 15%±2.5 vs. 7.6%±0.6, $p<0.01$). (Figure 20) However, PXR ligand rifampicin did not change the cell cycle distribution as compared to the DMSO control group.

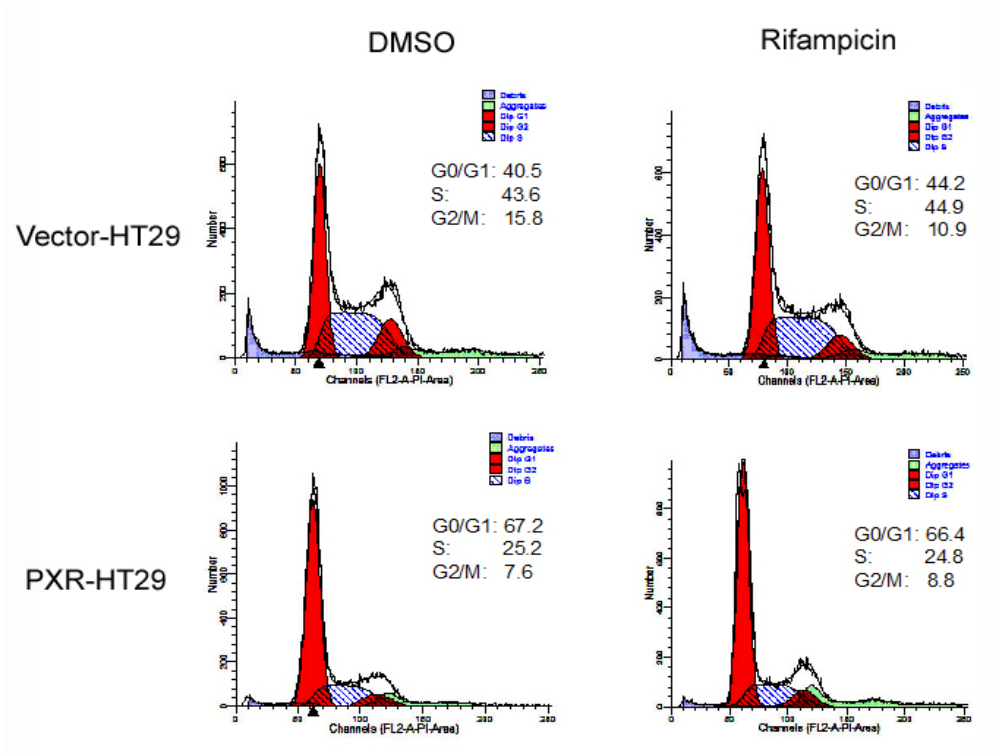

FIGURE 20: Results from flow cytometry indicate a G_0/G_1 arrest in PXR-HT29 cells. Cells transfected with PXR or vector were cultured up to 70% confluency, treated with DMSO or rifampicin for 24 hours and harvested by trypsinization. Cells were fixed in 70% ethanol, washed in PBS and resuspended in Propidium iodide (20 µg/ml) staining solution. Flow cytometry analysis was performed immediately in an FACS Calibur flow cytometer (Becton Dickinson) with an excitation at 488 nm and an emission at 620 nm. (Data was produced by Dr. Roger Smith)

PXR expression in colon cancer cells inhibited E2F1 and Rb expression

The Rb/E2F pathway is one of the most important pathways for progression through the G_1 phase of the cell cycle. When Rb is phosphorylated E2F is released from the combined complex and binds to DNA for transcriptional regulation. The result of immuno-histochemical staining in the xenograft tumors (corn oil treated tumors) showed that E2F1 is strongly expressed in Vector-HT29 tumors but only weakly expressed in PXR-HT29 tumors and is located in the nucleus of cancer cells (Figure 21A, upper

panel). There is a significant difference between the positive cell ratios of the two groups (Figure 21C, 35.0% ± 3.9 vs. 9.8% ± 1.9, $p<0.01$). Rb expression is also located in the nucleus of cancer cells (Figure A lower panel) and markedly decreased in PXR-HT29 tumors as compared to Vector-HT29 (Figure 21D, 26.2% ± 5.7 vs. 75.6% ± 10.4, $p<0.01$). Western blot analysis further confirmed the differential expression of E2F1 and Rb in the tumors from both groups (Figure 21B).

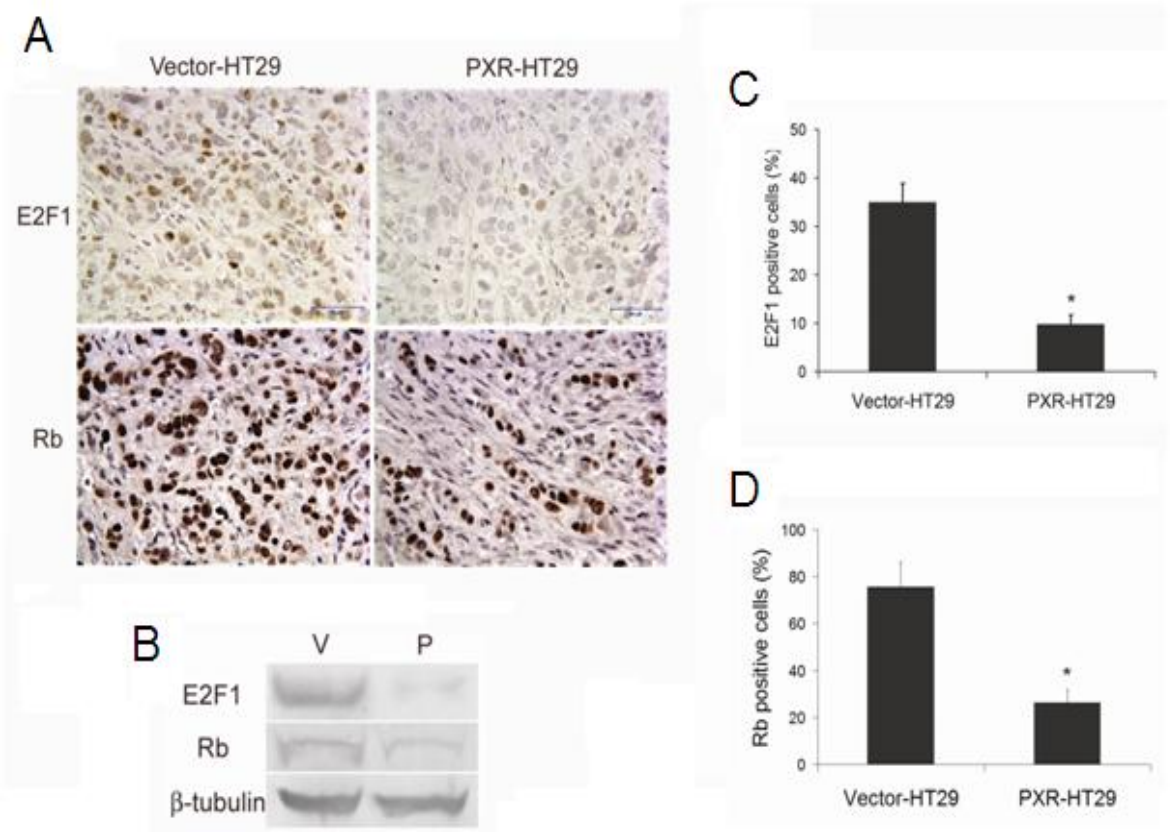

FIGURE 21: Analysis of xenograft tissues indicate that E2F1 and Rb levels are decreased in PXR positive tumors. Immuno-histochemical staining was performed in HT-29 tumors removed from the nude mice. Paraffin-embedded sections were deparaffinized, rehydrated, and microwave heated for antigen retrieval. After 15 minutes of blocking with normal serum, the primary antibody or corresponding control isotype IgG were applied and incubated overnight at 4°C. Slides were washed and the biotinylated secondary antibody and the streptavidin-biotin complex were applied, each for 30 minutes at room. After rinsing with PBS, the slides were immersed for 10 minutes in 3,3'-diaminobenzidine solution, monitored under microscope and the reaction was stopped with distilled water, then counterstained with hematoxylin, dehydrated, and coverslipped. Western blot was performed to confirm the presence of proteins seen in immuno-histochemical staining. Anti-β-tubulin was used as the protein level control. (Immuno-histochemistry conducted by Nengtai Ouyang)

DISCUSSION AND CONCLUSION

The human body deals with xenobiotics and drugs in an evolutionarily conserved process and is continuously shaped by gene-environment interaction. The human population continues to grow which increases our need for medications to combat disease and thus a more complete understanding of drug-drug interactions and adverse effects is required. Cytochrome P450 3A4 is a key drug metabolizing enzyme that is responsible for metabolism of over fifty percent of clinical medications. The pregnane X receptor, a member of the nuclear receptor super family, is a transcriptional regulator of CYP3A4 expression. PXR responds to endogenous and exogenous ligands. Our laboratory has been investigating the mechanism of PXR-regulated gene expression.

The pregnane X receptor directly interacts with CNOT2 to affect cytochrome P450 3A4 expression

In order to determine protein partners of PXR we screened the human cDNA library by yeast two-hybrid assay. Over one million independent clones were screened. One of the positive clones was a partial fragment of the C-terminal end of CNOT2. CNOT2 is the second and smallest protein component of the CCR4-NOT complex. The CCR4-NOT complex is an evolutionary conserved complex involved in most biological processes due to the promiscuity of protein interactions that the components of the complex conducts. One important function of the CCR4-NOT complex is RNA modification through deadenylation of poly(A) tails. However, the NOT components of

the complex are not responsible for this function; Traditionally the NOT components are involved in transcriptional regulation as co-activators or co-repressors.

Previously published reports indicate the NOT box and conserved domains of CNOT2 are the domains responsible for protein interactions (Albert *et al,* 2000; Zwartjes *et al,* 2006). We were able to verify this with respect to PXR. PXR ligand binding domain directly interacts with the NOT box and conserved domains of CNOT2. To verify the interaction in mammalian cells we performed mammalian two-hybrid assay and co-immunoprecipitation assay. Luciferase reporter gene assay and CNOT2 siRNA knock down indicate that when PXR-CNOT2 interact. These results demonstrate that PXR and CNOT2 interact to suppress PXR-regulated gene expression.

We have attempted to verify the location of PXR-CNOT2 interaction. Previously published literature indicated that components of CCR4-NOT complex colocalized in Cajal bodies (Wagner *et al,* 2007). Cajal bodies are dynamic structures that form when transcription is occurring. We were unable to colocalizes PXR protein and coilin protein due to the over expression of PXR. When PXR is over expressed the entire nucleus is stained for the target protein. However, we know that PXR can be found in both the nucleus and cytoplasm, since the general mechanism of action for PXR involves ligand activation in the cytoplasm and subsequent translocation into the nucleus. We should have tested cytoplasmic bodies also, for example GW-182 or Dcp16 bodies to determine the precise location of PXR-CNOT2 interaction. It is unlikely that PXR and CNOT2 interact within both the nucleus and cytoplasm.

In yeast, CCR4-NOT is the complex primarily responsible for RNA modifications. We hypothesized that there might be a role for PXR-CNOT2 in this aspect of RNA regulation. In order to test our hypothesis we performed poly(A) tail PCR with harvested RNA from HepG2 cells with and without PXR over-expression. There was little ligand effect but the results did indicate that less deadenylation occurred in cells containing PXR.

This is an interesting result because it contradicts the early result that PXR-CNOT2 interact to suppress PXR target gene transcriptional activity. It is possible that the cell is trying to expend the least amount of energy and in doing so PXR-CNOT2 has a two-sided job. Involvement in RNA modification could be a possible feedback mechanism that regulates expression but maintains protein production. CYP3A4 expression is suppressed at the transcriptional level, but the cell wants to maintain translation of the transcripts that were already produced. Due to the importance of CYP3A4 enzyme activity the cell does not want to completely repress expression of CYP3A4 transcripts.

Due to restrictions of our lab we were unable to investigate the effects on other PXR target genes such as multidrug resistance transporter 1 (MDR1) or other cytochrome P450s that influence drug transport and metabolism. It is possible that PXR-CNOT2 interact at the promoter region to direct splicing of cyp3a. In order to test this CNOT2 could be knocked down by siRNA and realtime PCR performed to determine the amounts of CYP3A isoforms, CYP3A4, 3A5, 3A7.

In conclusion, the first part of experiments conducted in this thesis research indicates a new role for PXR beyond transcriptional regulation of its target genes. We demonstrated that PXR has a role in post-transcriptional regulation of gene expression through interaction with component NOT2 of the CCR4-NOT complex to preserve stability of CYP3A4 mRNAs through modulation of deadenylation.

The pregnane X receptor suppresses colon cancer growth through interaction with proteins involved in cell cycle progression

The second part of the presented research demonstrates that PXR is influential in colon cancer progression. Colorectal cancer on average costs a patient $29,196, this includes treatment costs, doctor and hospital fees and other costs (Luo *et al,* 2010). With increased colon cancer incidence and mortality it is more important now to determine the mechanisms that contribute to colon cancer progression. Clinical efficacy of chemotherapy in colorectal cancer is subjected to broad inter-individual variations leading to the inability to predict outcome and toxicity; a substantial part of the variability observed among patients might be caused by increased expression of PXR which results in increased drug metabolism (Raynal *et al,* 2010).

Dia *et al.* (2008) showed that PXR is required for normal liver regeneration. From their results the underlying assumption that PXR might be involved in colon cancer led us to investigate the role for PXR as a tumor suppressor protein. Our initial results showed that transfected PXR gene inhibits cancer cell growth. Hence, the tumor suppressor activity of PXR was hypothesized and further investigated in HT29 colon cancer cells *in vitro* and *in vivo*.

The distribution of PXR in the nucleus of most cancer cells is consistent with other results that were found in human breast cancer tissue and may implicate functional activation of PXR. Initial observations made during routine cell culture indicated that cancer cells with PXR expression grew slower. HepG2 (liver carcinoma cells) and HT29 (colon carcinoma cells) with PXR stable transfection were 3-4 days behind reaching confluency of cells without PXR expression.

Separate distribution of PXR and Ki-67 further directly confirmed the inhibitory activity of PXR to proliferation in HT29 cells. Some studies on PXR have been involved in cell proliferation but this effect was mostly considered to be indirectly conducted by enhancing detoxification capability of the cells which then decreases cell proliferation by metabolizing the chemicals that promote cell proliferation. Here we report that PXR itself directly inhibits colon cancer cell proliferation or tumor growth because the principle enzymes that PXR regulates like CYP3A4 and CYP1A1 were not up-regulated after PXR transfection (data not shown).

In conclusion, these results may suggest that the induction of PXR over-expression in colon cancer cells has potential to be a new approach for tumor therapy. Our results suggest that PXR plays a novel biological function of anti-proliferation *in vitro* and *in vivo* beyond regulation of enzymes responsible for metabolism and detoxification. This function may be caused directly by inhibiting cell proliferation through Rb/E2F pathway in G_1 phase of cell cycle.

In order to apply our laboratory's research that PXR expression suppresses tumor growth one would need to weigh the differences in benefits of the available treatments.

Our results might be better suited to be applied as a preventive measure prior to identification of colorectal cancer and treatment by chemotherapeutics. For example, St. John's Wort is an effective ligand of PXR and therefore could be successfully utilized to increase PXR expression, in affect to suppress pre-cancerous colon cells. Yet again the problem arises of drug-drug interactions; if you are taking any other medications that are metabolized through PXR target genes taking a PXR ligand would also increase the metabolism of that medication.

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